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14. ABSTRACT: The role of tumor-associated neutrophils (TANs) in cancer progression remains unclear and has only been recently investigated in murine tumor models. However, there are limited data about the TANs in human tumor. The goal of this study is to provide a phenotypic and functional characterization of TANs in lung cancer patients. We have identified two major subsets of TANs in lung tumors: "canonical" TANs that express classic neutrophil markers and "APC-like hybrid" TANs that display a combination of canonical neutrophil markers and markers of antigen-presenting cells. In this study we have performed analysis of key inflammatory factors secreted by canonical and hybrid neutrophils. We have also performed whole human genome RNA expression profiles of these neutrophil subsets. We found that the APC-like hybrid neutrophils are superior to canonical neutrophils in their ability to trigger and support T cell responses in direct cell-cell interactions. This property of hybrid neutrophils may provide new opportunities to boost the efficacy of vaccines based on cytotoxic T lymphocyte induction. Our study for the first time provide a comprehensive functional characterization of TAN subsets in lung cancer patients and bridge the knowledge gap between prior data from murine studies to the human scenario.

15. SUBJECT TERMS human lung cancer, human tumor microenvironment, phenotype and function of tumor-associated neutrophils and their subsets, tumoricidal neutrophils, tumor inflammation, anti-tumor neutrophils, anti-tumor innate immune response, anti-tumor adaptive immune response, neutrophil and T cell interaction.

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INTRODUCTION

Subject: Lung cancer is the most common cause of cancer death amongst Veterans in the United States. Despite advances in therapeutic strategies, patients with lung cancer have a poor prognosis and the overall survival rate is less than 20% for all stages. Immunotherapy represents an attractive approach in the treatment of lung cancer; however previous cancer vaccines have been unsuccessful, likely due to the failure to address the influence of the tumor microenvironment. To date, the characterization of the human lung tumor microenvironment is still in its infancy and the functional cross-talk between immune and tumor cells in humans remains largely unexplored. Tumor-recruited myeloid cells represent a significant portion of inflammatory cells in the tumor microenvironment and influence nearly all steps of tumor progression. Among these cells, TANs represent a predominant cell type. The role of tumor-associated neutrophils (TANs) in cancer progression remains unclear and has only been recently investigated in murine models. However, virtually nothing is known about the phenotype and function of TANs in lung human tumors. Our study for the first time provide a comprehensive functional characterization of tumor-infiltrating neutrophil subsets in early-stage lung cancer patients and will bridge the knowledge gap between prior data from murine studies to the human scenario. We study a large cohort of veterans that were diagnosed with lung cancer in the Philadelphia VA Medical Center.

Purpose and scope of the research We have identified two major sub-populations of TANs in freshly isolated tumors from Non-Small Cell Lung Cancer (NSCLC) patients with Stage I-II. One subset of "canonical" TANs expressed classic neutrophil markers $CD11b^{+}Arg1^{+}MPO^{+}CD66b^{+}CD15^{hi}$. A second subset of TANs displayed a combination of canonical neutrophil markers plus markers ($CD14^{+}HLA\text{-}DR^{+}CCR7^{+}CD86^{+}$) normally expressed on antigen-presenting cells (APC). We termed this unique neutrophil population "APC-like hybrid TANs". The purpose of this proposal is to determine the specific roles of these distinct subsets of tumor-associated neutrophils in human lung cancers and to develop novel approaches to boost anti-tumor immunity. Specifically, (1) investigate the inflammatory profile of canonical and hybrid TANs, (2) investigate the effects of canonical and hybrid TANs on T cell responses and on the maturation and function of dendritic cells, and (3) define the cytotoxic phenotype of canonical and hybrid neutrophils and the mechanisms by which these neutrophils inhibit tumor growth.

KEYWORDS

human lung cancer, human tumor microenvironment, tumor-associated neutrophils, tumoricidal neutrophils, tumor inflammation, anti-tumor neutrophils, anti-tumor innate immune response. anti-tumor adaptive immune response, neutrophil and T cell interaction.

ACCOMPLISHMENTS

There were no significant changes in the project. We have performed all experiments according to plan proposed in the original proposal and approved SOW.

What were the major goals of the project?

The goal of this proposal is to determine the specific role of distinct subsets of tumor-associated neutrophils (TAN) in non-small cell lung cancer. Specifically:
Aim 1 goal: Investigate the inflammatory profile of canonical and hybrid TANs, (timeline 1-12 months).
Aim 2 goal: Investigate the effects of canonical and hybrid TANs on T cell responses and on the maturation and function of dendritic cells, (timeline 1-18 months).
Aim 3 goal: Define the cytotoxic phenotype of canonical and hybrid neutrophils and the mechanisms by which these neutrophils inhibit tumor growth, (timeline 12-24 months).

What was accomplished under these goals?

Aim 1. Investigate the inflammatory profile of canonical and hybrid TANs. This aim is completed

Specific objectives: The cytokines and chemokines produced by TAN subsets within the lung tumor are critical in mediating their effects on the tumor microenvironment. The purpose of this Aim was to characterize the inflammatory profile of canonical and hybrid TANs. Using transcriptomics and protein-based approaches we aimed to quantify key secreted inflammatory cytokines, chemokines, and growth factors secreted by canonical and hybrid TANs. In addition we performed whole human genome RNA expression profiles (in the Penn Molecular Profiling Facility) of TAN subsets.

Major activities: We have been able to isolate TANs and TAN subsets using flow cytometry sorting. Given that frequency of hybrid TANs in tissue is extremely low, the number of these sorted cells was relatively small to perform good quality multi-analyte analysis and whole human genome RNA expression profiles. We used all sorted hybrid TANs for functional assays proposed in Aim 2. To obtain larger numbers of cells for these studies, we took advantage of our BM neutrophil model described in our original proposal. Using this model we have performed analysis of key inflammatory factors secreted by canonical and hybrid neutrophils (please see 1st year annual report). Over the first year of award we have also performed whole human genome RNA expression profiles of these neutrophil subsets. During the second year of proposal in collaboration with the University of Pennsylvania Next Generation Sequencing Core Facility, and the Upenn Bioinformatics Core, we were able to perform top-level analysis of our data and obtained the genetic landscape for both canonical and hybrid neutrophils. Furthermore, in collaboration with biostatisticians performed gene-annotation enrichment analysis, functional annotation clustering, and translational targets to define the unique features of hybrid neutrophils. See all details below.

Significant results and outcomes: To obtain sufficient numbers of hybrid and canonical neutrophils cells for this study we have identified conditions in which the immature bone marrow granulocytes could be differentiated into cells that phenotypically and functionally recapitulate canonical TANs or TAN hybrid cells. We have justified the use of this new model of bone marrow-derived canonical and hybrid neutrophils to comprehensively investigate the rare subset of hybrid TANs (Singhal et al., Cancer Cell, 2016). For more details see our published paper and 1st year annual report (Singhal et al., Cancer Cell, 2016). Using this model we have performed analysis of key inflammatory factors secreted by canonical and hybrid neutrophils (please see 1st year annual report).

To more fully characterize these cells in a non-biased fashion and to allow us to look at many other pathways, the canonical and hybrid BM derived neutrophils were flow sorted and immediately placed into RNAlater® Stabilization Solution. mRNA were isolated using the Qiagen RNAeasy micro kit from sorted HLA-DR^{+}CD14^{+} Double Positive (DP) hybrid and HLA-DR^{-}CD14^{-} Double Negative (DN) canonical neutrophils. Whole human

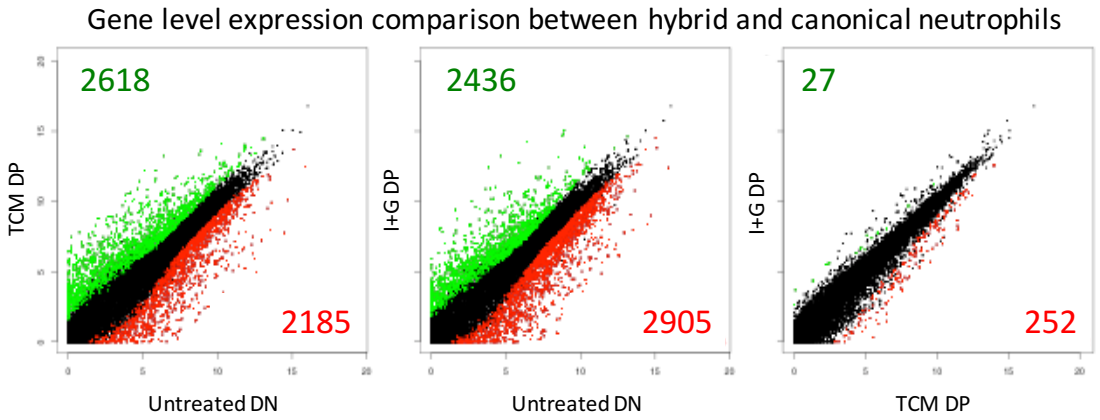
genome RNA expression profiles have been performed using RNA next generation sequencing (Penn Molecular Profiling Facility). Our primary analysis correlating RNAseq data to flow cytometry data has provided confidence that in-depth analysis will reveal valid transcritpional targets for interrogation. We observed a strong correlation between gene expression and our previous published surface protein data. An analysis of the quality of the reads showed that the intra-sample variability was low, suggesting similar gene clusters were induced within treatment groups. These initial findings impart confidence that future analysis of any transcriptional differences between each subset will yield meaningful targets (please see 1st year annual report). During this year of proposal we were able to perform top-level analysis of our data and obtained the genetic landscape for both canonical and hybrid neutrophils (Figures 1-3).

A principle component analysis comparing the canonical DN to hybrid DP subsets, either expanded through co-culture with tumor conditioned media (TCM DP) or interferon gamma/GM-CSF treatment (I+G DP), revealed some interesting differences in the overall transcriptional profiles (Figure 1A). A comparison of the differentially expressed genes between DN canonical and TCM-induced DP hybrid cells show that there are 2618 genes increased in DP versus 2185 unique genes that are differentially expressed in the DN canonicals. These data reveal that while there are numerous overlapping genes expressed, there are a substantial number of genes that differ between the two subsets. It is here that we are focused on gaining insights into the molecular pathways that are necessary and sufficient for progression into the hybrid neutrophil lineage. We also noted similar numbers of genes expressed between the I+G DP and TCM DP hybrid subsets, suggesting that while different conditions are used to generate the hybrid neutrophils, the hybrids are a unique population with distinct set of genes as revealed by genetic analysis. We are currently evaluating whether the larger number of genes differentially expressed within the I+G DP subset are due to exposure to the IFNg/GM-CSF, or represent bona fide lineage commitment pathways. Given that multiple genes have IFN responsive elements, it will take some time to determine the answer.

Ingenuity Pathway Analysis software was used to analyze the top canonical pathways utilized by each DP subset. Both the I+G DP and TCM DP hybrid neutrophils share increased usage of Th1 and antigen presentation pathway genes as compared to the DN canonical neutrophil subsets (Figure 1B). As we had previously observed an increase in antigen presentation molecules by flow cytometry, and the IPA software revealed that both the TCM DP and I+G DP hybrid neutrophils had significantly expressed genes within this pathway (p-value 1.41 e-05 and 1.47 e-04 respectively). We used the software to analyze the log2 expression of the genes using the Untreated DN cells as a baseline. In total, the genes of the antigen presentation pathway are expressed to a similar extent in both DP–derived subsets (Figure 1C). Our more detailed analysis of the top 100 differentially expressed genes within the DP hybrid subset reveals multiple overlapping genes that are shared with both the I+G DP and TCM DP hybrid populations (Figure 2A). Consistent with the IPA software results, we observe a molecular signature of antigen presentation cells, as MHC-Class II family members (HLA-DP, HLA-DR, CIITA) and co-stimulatory molecules (CD40, ICOSL) show increased expression (Figure 2B). We rank-ordered the top 50 upregulated and downregulated genes, then built a heatmap to allow visualization of each patient's RNAseq data (Figure 2C). As seen in the heatmap, the genes up regulated and down regulated are largely similar across patient samples, further providing confidence that the genes affected are critical for adoption of the hybrid neutrophil fate. In trying to ascribe a unified functionality to the genes expressed, the IPA software revealed top KEGG pathways utilized in shared genes (Figure 2D). The KEGG pathways are pathway maps that represent known molecular interactions for multiple cellular functions, such as metabolism and human diseases. We are currently investigating these pathways to see if there are clues to genetic signatures that correlate with the hybrid neutrophil subsets. Figure 2E reveals a map of connected and overlapping canonical pathways as determined by the IPA software. These are also being investigated to determine if a specific node is necessary and sufficient to drive development into the DP pathway. We will require additional time to explore these insights, which are beyond the scope of this proposal. Finally, as Thrombospondin-1 was the top up-regulated gene in TCM DP hybrid cells, we decided to examine mRNA expression in patient samples that had been differentiated to DP hybrid neutrophils (Figure 3). Strikingly, we found the Thrombospondin-1 (Tsp-1) is significantly upregulated in all patient bone marrow neutrophils differentiated to hybrids using TCM. We are currently working to produce loss-of-function conditions in human neutrophils by using adeno-viral

mediated transduction. The relevance of Tsp-1 in cancer and neutrophil biology is partially established, as it is known to be expressed in neutrophils and regulate anti-tumor responses via suppression of VEGF.

A



B

Untreated DN to TCM DP

Top Canonical Pathways		
Name	p-value	Overlap
Th1 Pathway	6.67E-08	51.5 % 67/130
Th1 and Th2 Activation Pathway	2.14E-06	45.6 % 82/180
Antigen Presentation Pathway	1.41E-05	63.2 % 24/38
Dendritic Cell Maturation	2.86E-05	43.4 % 79/182
Cholesterol Biosynthesis I	5.42E-05	84.6 % 11/13

Untreated DN to I+G DP

Top Canonical Pathways		
Name	p-value	Overlap
Fc Receptor-mediated Phagocytosis in Macrophages and Monocytes	2.32E-06	51.6 % 48/93
Th1 Pathway	3.18E-05	45.4 % 59/130
Actin Cytoskeleton Signaling	7.81E-05	40.5 % 90/222
Th1 and Th2 Activation Pathway	1.06E-04	41.7 % 75/180
Antigen Presentation Pathway	1.47E-04	57.9 % 22/38

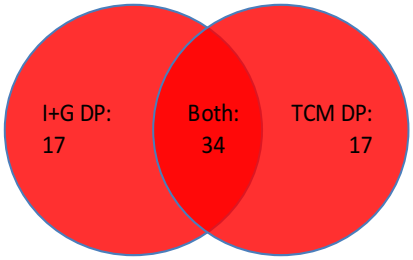
C

IPA Antigen Presentation Pathway Gene Use

Symbol	Entrez Gene Name	Gene symbo	TCM DP Expr Log Ratio	I+G DP Expr Log Ratio
B2M	beta-2-microglobulin	B2M	-1.386	-1.181
CALR	calreticulin	CALR	-1.636	-1.055
CD74	CD74 molecule	CD74	-4.757	-5.513
CIITA	class II major histocompatibility complex transactivator	CIITA	-5.795	-7.031
MR1	major histocompatibility complex, class I-related	MR1	-1.175	
HLA-A	major histocompatibility complex, class I, A	HLA-A	-1.197	-1.047
HLA-G	major histocompatibility complex, class I, G	HLA-G	-1.148	
HLA-DMA	major histocompatibility complex, class II, DM alpha	HLA-DMA	-5.605	-6.074
HLA-DMB	major histocompatibility complex, class II, DM beta	HLA-DMB	-6.343	-6.935
HLA-DOA	major histocompatibility complex, class II, DO alpha	HLA-DOA	-2.759	-5.238
HLA-DOB	major histocompatibility complex, class II, DO beta	HLA-DOB	-2.688	-2.273
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	HLA-DPA1	-6.937	-8.431
HLA-DPB1	major histocompatibility complex, class II, DP beta 1	HLA-DPB1	-6.747	-8.319
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	HLA-DQA1	-6.371	-7.788
HLA-DQA2	major histocompatibility complex, class II, DQ alpha 2	HLA-DQA2	-2.084	-6.187
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	HLA-DQB1	-5.589	-5.953
HLA-DQB2	major histocompatibility complex, class II, DQ beta 2	HLA-DQB2	-3.914	-5.79
HLA-DRA	major histocompatibility complex, class II, DR alpha	HLA-DRA	-7.174	-7.756
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	HLA-DRB1	-7.004	-7.383
HLA-DRB5	major histocompatibility complex, class II, DR beta 5	HLA-DRB5	-5.952	-6.323
NLRC5	NLR family CARD domain containing 5	NLRC5	1.071	
PSMB5	proteasome subunit beta 5	PSMB5	-1.394	-1.187
PDIA3	protein disulfide isomerase family A member 3	PDIA3	-1.402	
TAP2	transporter 2, ATP binding cassette subfamily B member	TAP2	-1.809	-1.933

Figure 1. APC-like hybrid neutrophils have a gene expression profile distinct from canonical neutrophils
(**A**) Gene level expression comparison between hybrid (TCM DP and I+G DP) and canonical neutrophils (DN). Plots correlate all relative genes expressed between indicated neutrophil subsets. Dots in green or red indicate differentially expressed genes (false discovery rate of >5%and fold change >1) with the total numbers shown in the upper left and lower right quadrants. (**B**) Top canonical pathways identified for hybrid neutrophils using Ingenuity Pathway Analysis (IPA) software. IPA identified and rank-ordered the expression of significantly expressed canonical pathways within the hybrid neutrophils subsets. Numbers show p-value and % overlap of up regulated genes defining the particular pathway. (**C**) Hybrid neutrophil gene expression within the Antigen Presentation Pathway. Expression of the genes defined by the IPA software to be within the Antigen Presentation Pathway. Table shows the gene name, symbol, and expression as log2 for both the I+G DP and TCM DP hybrid neutrophil subsets. Fold change is over canonical neutrophils.

A



B

Unique+IGDP	Unique TCM DP	Shared
HLA-DRB6	THBS1	HLA-DPB1
XAF1	ARID5B	HLA-DPA1
C2CD2	SERPINB2	HLA-DRA
PPARGC1B	SOCS3	APOL4
HLA-DQB2	KREMEN1	EPSTI1
RSAD2	SDC4	CIITA
HERC5	ABHD6	HLA-DMB
IFI6	RASSF4	OAS2
CDKN1A	SDC3	HLA-DRB1
STAT1	TMEM51	HLA-DMA
HLA-DQA2	TMEM106A	HLA-DRB5
BTN3A3	PTGR2	DDX60
LGALS3BP	FAM198B	HLA-DQA1
CMPK2	LMNA	CD74
LAP3	HBEGF	SIGLEC1
TRIM22	AHNAK	ICOSLG
SLC11A2	PRKCA	ACP5
		SLC25A19
		CD40
		HLA-DQB1
		CD9
		RASGEF1B
		RASAL1
		SLAMF8
		ENG
		DAGLA
		C1orf204
		STON2
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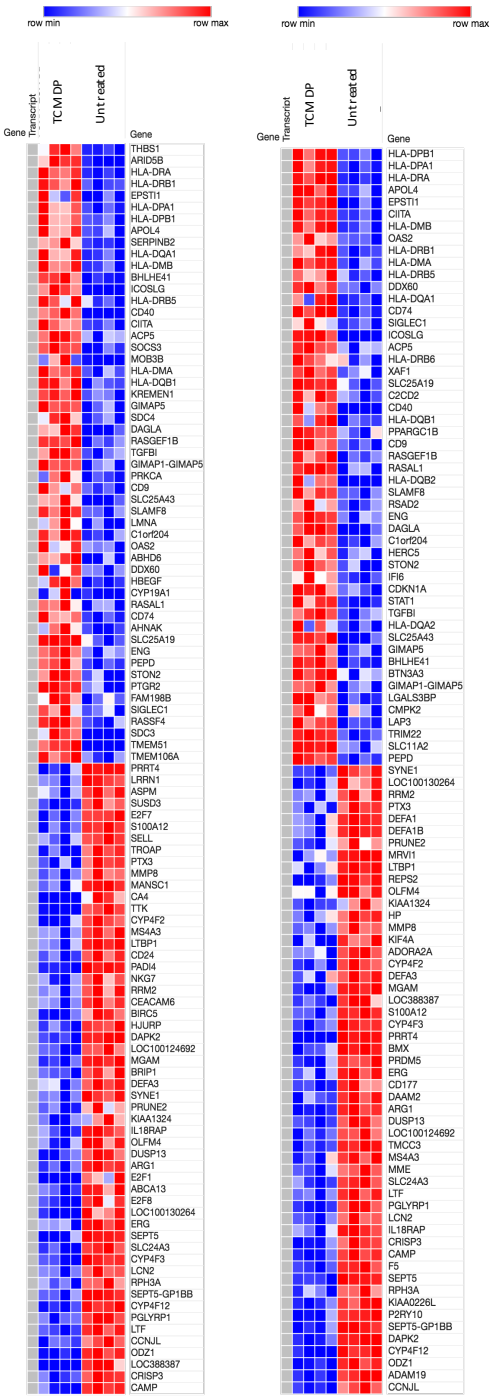
		SLC25A43
		GIMAP5
		BHLHE41
		GIMAP1-GIMAP5
		PEPD

C



D

Top KEGG Pathways identified

hsa04672:Intestinal immune network for IgA production
hsa05310:Asthma
hsa05330:Allograft rejection
hsa04612:Antigen processing and presentation
hsa05320:Autoimmune thyroid disease
hsa05332:Graft-versus-host disease
hsa05416:Viral myocarditis
hsa04514:Cell adhesion molecules (CAMs)
hsa04940:Type I diabetes mellitus
hsa05145:Toxoplasmosis
hsa05323:Rheumatoid arthritis
hsa05150:Staphylococcus aureus infection
hsa05321:Inflammatory bowel disease (IBD)
hsa05140:Leishmaniasis
hsa05164:Influenza A
hsa05152:Tuberculosis
hsa05168:Herpes simplex infection
hsa05322:Systemic lupus erythematosus
hsa04145:Phagosome
hsa05166:HTLV-I infection

E

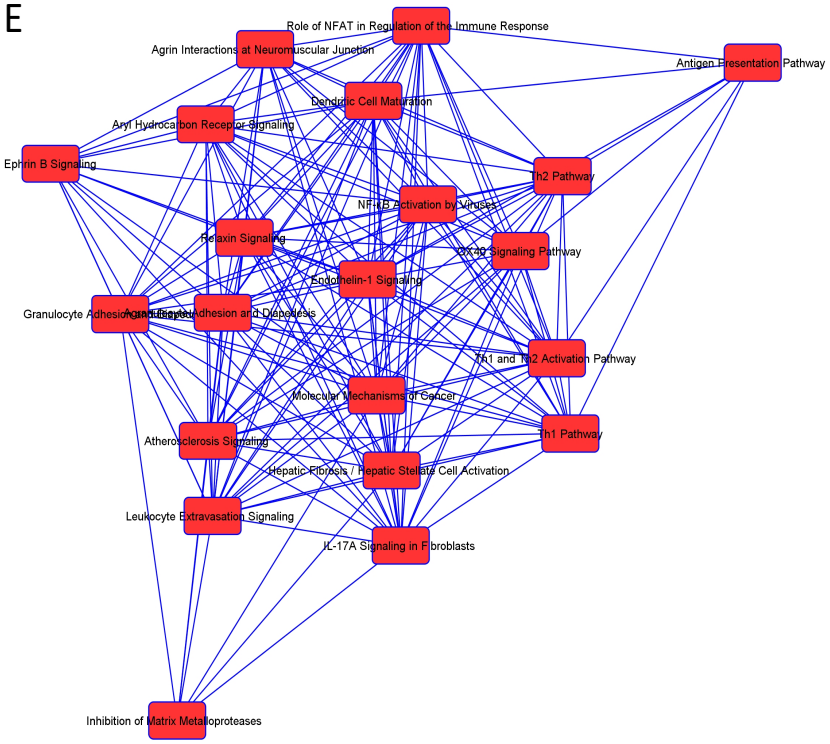


Figure 2. APC-like hybrid neutrophils have a gene expression profile distinct from canonical neutrophils
(**A**) Venn diagram showing the number of unique or overlapping genes differentially expressed in the hybrid neutrophil subsets. The top 100 genes within hybrid neutrophils that were differentially up regulated over canonical neutrophils were identified and compared between TCM DP and I+G DP subsets.
(**B**) Table identifying the top 100 genes that are up regulated over canonical neutrophils and are either unique to I+G DP, TCM DP, or are shared by both. The individual gene symbols are shown.
(**C**) Heat maps showing relative expression levels for each of the top 50 up regulated and top 50 down regulated genes. Each block within a row shows the relative gene expression for a single patient for the identified gene. Red indicates increased expression and blue indicates reduced expression.
(**D**) Top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for hybrid neutrophils. The KEGG are a collection of databases dealing with genomes and biological pathways. The IPA software flagged several KEGG pathways as being preferentially utilized in the hybrid neutrophils.
(**E**) IPA software analysis of overlapping canonical pathways in hybrid neutrophils. Overlapping canonical pathways that were significantly expressed within hybrid neutrophil subsets are shown above. Each node corresponds to a canonical pathway detected as significant and lines between the nodes highlight relationships between the pathways.

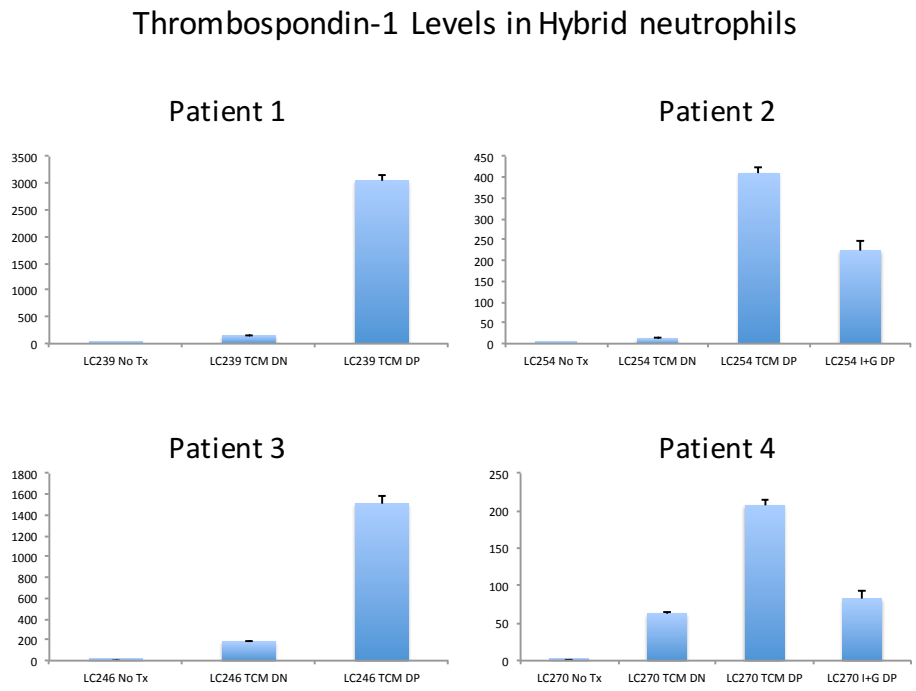


Figure 3. Thrombospondin-1 expression is increased in patient-derived hybrid neutrophils. mRNA expression of the human thrombospondin-1 gene were measured by real time PCR. Bar graphs indicated fold change of thrombospondin-1 for four individual patients.

Conclusions: Thus we defined and quantified key secreted inflammatory cytokines, chemokines, and growth factors secreted by canonical and hybrid neutrophils. In addition we performed whole human genome RNA expression profiles of these two populations of neutrophils. Based on these data we have proved that subset of hybrid neutrophils is a new population of neutrophils with its unique gene expression profile distinct from canonical neutrophils. The most upregulated genes in hybrid population are genes of antigen presentation and T cell stimulation pathways indicating that neutrophils are plastic and can acquire characteristics of professional APC.

Aim 2. Investigate the effects of canonical and hybrid TANs on T cell responses and on the maturation and function of dendritic cells. This aim is completed

Specific objectives: Goal of this aim was to evaluate the interaction of canonical and hybrid TANs with T cells in patients with NSCLC. Specifically, investigate the effects of TAN subsets on the antigen non-specific and specific T cell responses. Given that hybrid TANs exhibit many of the essential features of professional antigen presenting cells, we hypothesized that this hybrid TAN subset can trigger and augment T cell responses as compared to canonical TANs (aim 2.1) Understanding the role of TANs in regulating T cell responses in cancer patients is particularly important because cytotoxic T lymphocytes are the chief effector cells mediating antigen-driven anti-tumor immunity. We also investigated whether TANs "license" DCs towards immunogenic or tolerogenic cells (aim 2.2)

Major activities: Aim 2.1. During the first year of proposal we determined the effect of canonical and hybrid neutrophils on antigen non-specific and specific T cell responses. For more details see our published paper and 1st year annual report (Singhal et al., Cancer Cell, 2016). During the second year of proposal we performed experiments as originally described in aim 2.2 and investigated the role of TANs in cross-talk with dendritic cells (DCs). Given that frequency of hybrid TANs in tissue is extremely low, the number of these sorted cells was relatively small to perform these functional assays we used our BM neutrophil model described in our original proposal. We have justified the use of this new model of bone marrow-derived canonical and hybrid neutrophils to comprehensively investigate the rare subset of hybrid TANs (Singhal et al., Cancer Cell, 2016). For more details see our published paper and 1st year annual report (Singhal et al., Cancer Cell, 2016). Thus we co-cultured monocyte-derived DCs (MoDCs) with bone marrow derived canonical or hybrid neutrophils and measured the (i) maturation of these co-cultured DC after stimulation with LPS, (ii) the cytokine production (IL-6, IL-10, IL-12, TNF-a). In addition we evaluated the ability of these co-cultured DC to trigger proliferation of allogeneic T cells in the mixed lymphocyte response (MLR).

Significant results and outcomes:

Aim 2.1 (Investigate the effects of TAN subpopulations on T cell responses). This aim is completed. We have rigorously tested our hypothesis and proved it correct during the first year of the project. Our team has published these results in the following journals: (Eruslanov, JCI, 2014), (Singhal, Cancer Cell, 2016), (Moon, Clinical Cancer Research, 2016). Briefly, we found that the APC-like hybrid neutrophils are superior to canonical neutrophils in their ability to: (1) stimulate antigen non-specific autologous T cell responses, (2) directly stimulate antigen-specific autologous memory T cell responses and (3) augment tumor-specific effector T cell responses by providing a co-stimulatory signals through the OX40L, 4-1BBL CD86, CD54 molecules in direct cell-cell contact. Together, we have proved that HLA-DR^+ hybrid TANs are able to function as efficient APCs and dramatically augment T cell response. For more details see our published papers and 1st year annual report.

Aim 2.2 (Determine the effects of TAN subpopulations on the maturation and function of dendritic cells). This aim is completed. We differentiated immature bone marrow neutrophils into canonical and hybrid neutrophils in vitro for 6 days using different TCM as earlier described (Singhal et al., Cancer Cell, 2016). We also isolated CD14 monocytes from the same patient and cultured them with IL-4 (20ng/ml) and GM-CSF (20ng/ml) (PeproTech) for 6 days to obtain the immature DC. At day 6 these immature DCs were co-cultured with or without different types of canonical and hybrid neutrophils in the presence of LPS (100ng/ml) for additional 24 hours. Next, we measured the (i) maturation of these co-cultured DC after stimulation with LPS, (ii) the intracellular cytokine production (IL-6, IL-10, IL-12, TNF-a) after stimulation with LPS (Figure 4 A and 4B). In addition we evaluated the ability of these co-cultured DC to trigger the proliferation of allogeneic T cells in the mixed lymphocyte reaction (MLR) (Figure 4C). We could not confirm our hypothesis and did not find any differences between canonical and hybrid neutrophils in terms their ability to affect (i) DC maturation, (ii) the

intracellular production of IL-6, IL-10, IL-12, TNF-a by DC, and (ii) the DC-triggered mixed lymphocyte response. (Figure 4A-C). Also we found that the proliferating allogeneic T cells in the MLR with different DC were able to produce the same level of IFN-g. We could not find the substantial level of IL-17 production in our system (data not shown). These data suggest that DC co-culture with either canonical or hybrid neutrophils trigger the same Th1-oriented T cell response. However, we found that DC co-cultured with either canonical or hybrid neutrophils were less responsive in DC maturation assay compared to control DC that had not been co-cultured with neutrophils (Figure 4A). We believe that this negative effect of differentiated neutrophils on DC maturation was mostly due to the presence of dying neutrophils in DC/neutrophil cell co-culture during stimulation with LPS, because the DC co-cultured with freshly isolated BMN from different patient did not show these dramatic changes in their activity to mature and trigger MLR. Importantly to note that even if we observed decreased phenotypic maturation of co-cultured DC, function of these cell (cytokine production and MLR) was unchanged (Figure 4A-C).

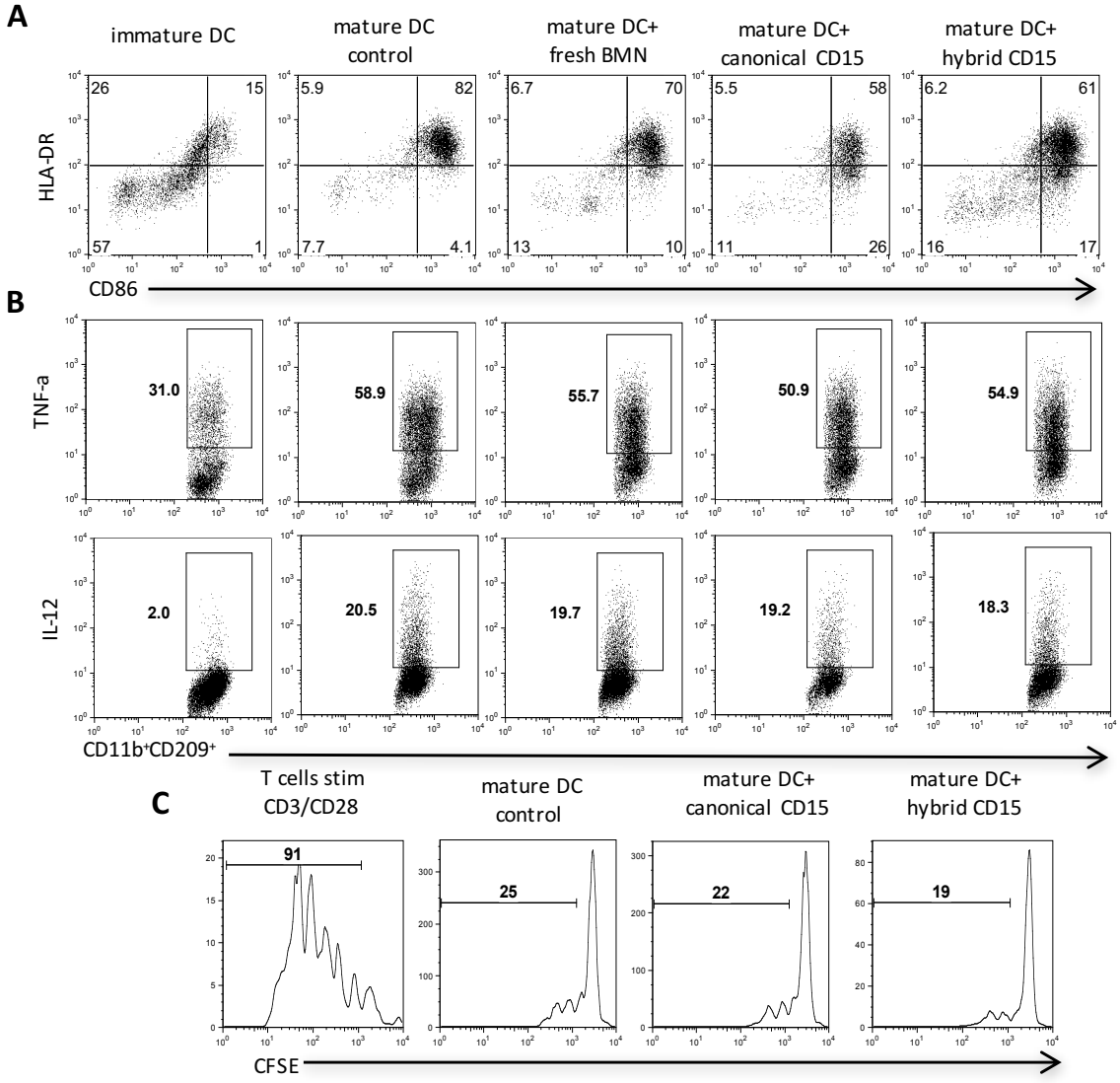


Figure 4. Effects of neutrophil subpopulations on the maturation and function of dendritic cells.
(**A**) Monocyte-derived DC were co-cultured with canonical and hybrid neutrophils and than stimulated with LPS for 24 hrs. Maturation of DC was analyzed by flow cytometry on live CD11b+CD209+ cells. (**B**) Monocyte-derived DC were co-cultured with canonical and hybrid neutrophils and than stimulated with LPS for 24 hrs. Intracellular cytokine production was analyzed by flow cytometry on live CD11b+CD209+ cells. (**C**) Monocyte-derived DC were co-cultured with canonical and hybrid neutrophils, stimulated with LPS and than purified by depleting all CD15 neutrophils from these co-cultures. T cells were purified from healthy donor volunteer using T cell enrichment columns. Purified T cells were labeled with CFSE and mixed with LPS stimulated DC for 5 days. Proliferation of T cells was analyzed in CFSE dilution assay.

Conclusions: The concept of neutrophil diversity and plasticity has begun to emerge in a variety of inflammatory disorders and murine tumor models; however, to date there has been no convincing evidence showing that specialized neutrophil subpopulations with different functions exist in human cancers. Thus, we provide the first evidence of two subsets of TANs in human lung cancer. All TANs have an activated phenotype and could support (rather than inhibit) T cell functions to some degree. However, we identified a subset of TAN in early-stage lung tumors that can undergo a unique differentiation process resulting in formation of specialized subset of APC-like hybrid neutrophils. These hybrid TANs had enhanced ability to trigger and support T cell responses in direct cell-cell interactions with minimum effect on DC functions. This property of hybrid neutrophils may provide new opportunities to boost the efficacy of vaccines based on cytotoxic T lymphocyte induction.

Aim 3: Define the cytotoxic phenotype of canonical and hybrid neutrophils and the mechanisms by which these neutrophils inhibit tumor growth. This aim is largely completed. To finish this aim we got a no-cost extension of this proposal without any changes in the approved objectives or scope of the project.

Specific objectives: Neutrophils are originally equipped with highly cytotoxic substances. Neutrophil-mediated tumor cytotoxicity has been demonstrated in vitro using activated neutrophils from the peripheral blood of healthy donors and cancer patients. However, there are no direct studies demonstrating the tumor-lytic function of human TANs. Thus, goal of this Aim was to determine spontaneous and tumor-specific Ab triggered tumoricidal activity of canonical and hybrid neutrophils.

Major activities: During this year we performed experiments according to our original plan described in the proposal. We evaluated spontaneous cytotoxic activity against tumor cells of the various subtypes of neutrophils (blood, BM neutrophils, BM-derived canonical and hybrid neutrophils and TANs). For this purpose we used GFP-expressing tumor cell line (lung carcinoma cells A549) as a target. These cells were incubated with the neutrophils for 24 hours, at which time all floating cells, including dying tumor cells, were removed. We calculated cytotoxicity as the remaining cell-associated GFP fluorescence of adherent tumor cells cultured with GFP-negative neutrophils to control wells (tumor cells without neutrophils). We have also determined whether TNF-α-or IFN-γ priming or pre-activation with PMA, anti-CD16/CD32, or fMLP can trigger the cytotoxic activity of neutrophil subsets. In addition, we optimized our flow cytometry-based approach to investigate the mechanisms by which neutrophils might mediate Ab-dependent cellular cytotoxicity against tumor cells. For these experiments we used different FcyR-bearing cells as a control cells with known ability to mediate antibody dependent cell cytotoxicity: (i) blood NK cells that are known to mediate antibody dependent cell cytotoxicity (ADCC) and (ii) monocyte derived macrophages that are known to mediate antibody dependent cell phagocytosis (ADP). These FcyR-bearing cells were co-cultured with cetuximab-opsonized A431 tumor large-size cells or rituximab-opsonized small-size Daudi that had been labeled with red-fluorescent dye PKH26. At the end of incubation, cells were collected and stained with FITC-Abs specific for NK (CD56) or Mo-Mph (CD14) and with a viability dye FVD eFluor® 660. Phagocyted PKH tumor cells were identified as both PKH^+ and $CD14^+$ cells by flow cytometry. Direct tumor cell death was calculated as a percent of $PKH^+FVD660^+$ CD56-CD14- cells that were not phagocyted.

Significant results and outcomes:

Figures 5A and B demonstrate that all tested populations of neutrophils, except hybrid neutrophils, were not able to spontaneously kill tumor cells. It appears that hybrid cells exert some spontaneous tumoricidal activity but at very low level. The priming of all types of neutrophils with TNF-a and IFN-g was also not sufficient to induce significant tumor cell death. Likewise, activation of neutrophils with CD16/CD32 Abs or fMLP did not result in significant induction of cytotoxic activity towards tumor cells. Importantly, however, we found that activation of all types of neutrophils with PMA caused dramatic tumor cell death. We found that the production of ROS by PMA activating neutrophils resulted in the massive tumor cell death because the inhibition of

NADPH oxidase complex by apocynin abrogated this strong tumoricidal effect (Figure 5A and 5B). Thus. we partially confirmed our hypothesis that hybrid neutrophils are able to spontaneously kill tumor cells although at very low level.

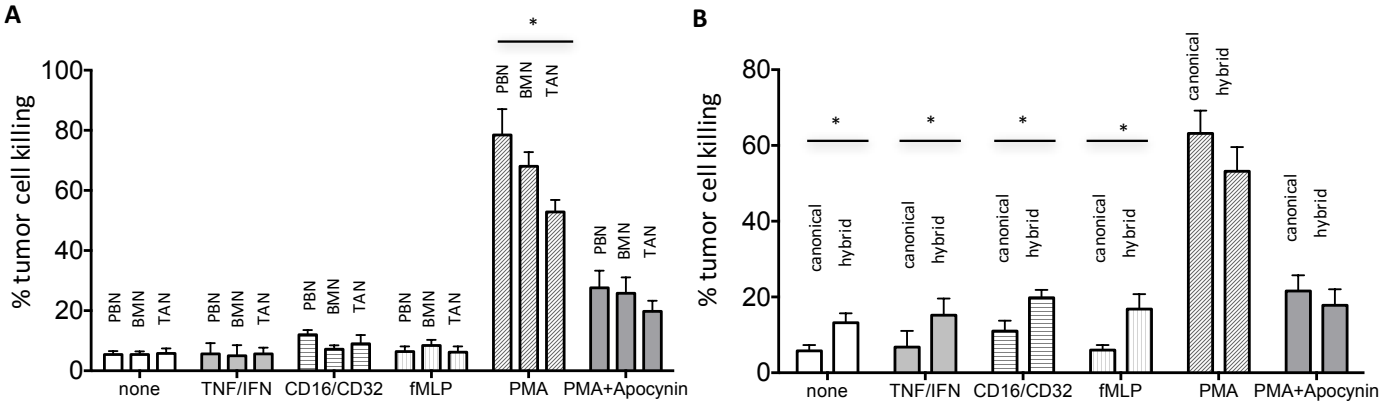


Figure 5. Cytotoxic activity of different neutrophil populations towards tumor A549 cell line
(A) PBN, BMN and TAN were isolated from the same lung cancer patient and mixed with GFP-expressing A549 tumor cells at a ratio 10:1(neutrophil:target). Twenty four hours later, floating cells, including dying tumor cells, were removed. The tumor cell killing was calculated as the remaining cell-associated GFP fluorescence of adherent tumor cells cultured with GFP-negative neutrophils to control wells (tumor cells without neutrophils). (B) Canonical and hybrid neutrophils were differentiated from bone marrow immature neutrophils and mixed with GFP-expressing A549 tumor cells at a ratio 10:1 (neutrophil:target). In some experiments neutrophils were primed or activated with indicated factors. Graphs represent the summary of five independent experiments. *$p<0.05$, Wilcoxon test for paired samples.

We have also optimized our flow cytometry-based approach to investigate the potential mechanisms by which different neutrophil populations might mediate Ab-dependent cellular cytotoxicity against tumor cells. Figure 6 clear shows that that our flow-based approach is able to clearly distinguish NK-mediated ADCC and macrophage-mediated ADP. Next we will determine the potential mechanisms by which different neutrophil populations mediate Abs triggered tumor cell cytotoxicity.

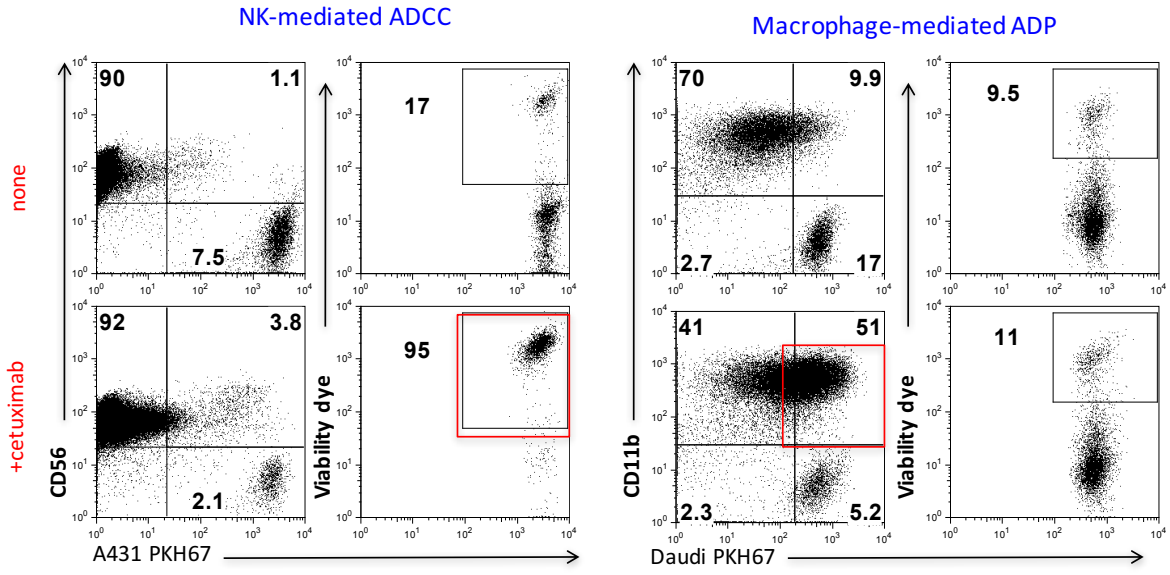


Figure 3. NK and Macrophage mediated tumoricidal activity towards opsonized tumor cells. (A) NK cells were isolated from PBMC with CD56 beads. (**B**) Mo-derived macrophages were differentiated from CD14 monocytes isolated from PBMC. CD56 NK cells were incubated with Cetuximab-opsonized A431 tumor cell line labeled with PKH67 (green) dye. CD11b macrophages were incubated with retuximab-opsonized Daudi tumor cell line labeled with PKH67 (green) dye. Eighteen hours later, cell cultures were collected and stained for viability (FVD660), CD56 (red), and CD11b (red) to visualize NK cells and macrophages. ADP was evaluated by flow cytometry as a percentage of PKH positive effector cells (red boxes). ADCC was evaluated as percent of FVD660 positive PKH labeled tumor cells (red boxes)

What opportunities for training and professional development has the project provided?

During the two years of award I have followed to my career development plan.
Specifically,
1. I have learned new research skills and expanded my research scope by taking the following:

- courses in the Supervisory Program that provides an introduction to the skills and information necessary to being an effective supervisor provided by University of Pennsylvania
- several grant writing seminars for junior faculty offered at University of Pennsylvania
- several scientific paper writing seminars for junior faculty offered at University of Pennsylvania
- weekly research seminars in immunology sponsored by the Penn Institute of Immunology and Penn Transplant Institute

2. I meet on a weekly basis with my mentor Dr. Albelda to monitor my scientific progress and ensure my career milestones are being met.

3. I participated in major immunology and cancer biology conferences sponsored by American Association for Cancer Research (AACR), American Association of Immunologists (AAI) and Society For Leukocyte Biology (SLB). As recognition of my research, I was invited several times to be a speaker and present results at the following conferences and seminars:

2017

- AAI Annual Meeting, Immunology 2017, May 12-16, Washington, D.C. **Invited Speaker**
- The Division of Transplant Immunobiology and the Department of Pathology and Laboratory Medicine at the Children's Hospital of Philadelphia. Philadelphia, September 20th, **Invited Speaker**
- Immuno-oncology seminar, Translational Genomics Research Institute, Phoenix, April 25-26. 2017, **Invited Speaker**
- Immuno-oncology seminar, AstraZeneca, Waltham, MA, 2017, March 16, **Invited Speaker**

2016

- E. Eruslanov. Tumor-Associated Neutrophils in Human Lung Cancer. Inflammation, Immunity and Cancer: The Society For Leukocyte Biology's 49th Annual Meeting and "Neutrophil 2016": Neutrophils and Other Leukocytes, University of Verona, Verona, Italy, September 15-17, 2016, **Invited Speaker**.
- E. Eruslanov. Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen Presenting Cell Features in Early-Stage Human Lung Cancer. Regulatory Myeloid Suppressor Cells Conference, Philadelphia, The Wistar Institute, June 16-19, 2016 **Invited Speaker**.
- E. Eruslanov. Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen Presenting Cell Features (Hybrid TANs) in Early-Stage Human Lung Cancer. Immunology School "Regulation of Lung Inflammation", Moscow, Russia, May11-13, 2016. **Invited Speaker**.
- Eruslanov, P Bhojnagarwala, J Quatromoni, S O'Brien, E Moon, T Stephen, A Rao, A Garfall, W Hancock, J Conejo-Garcia, C Deshpande, M Feldman, S Singhal and S Albelda. The origin and role of APC-like hybrid tumor-associated neutrophils in early-stage human lung cancer. AACR annual meeting: The function of tumor microenvironment in cancer. San Diego, Jan 7, 2016, **poster presentation**

Therefore, this career development award enables me to develop new research skills, knowledge and collaborations that dramatically advances my career as researcher of the human tumor microenvironment.

How were the results disseminated to communities of interest?
"Nothing to Report."

What do you plan to do during the next reporting period to accomplish the goals?

We requested 1-year no-cost extension because we were delayed in the initial onset of the project for IRB related issues. We will be completing the proposed Aim3 with no modifications. Please see the attached letter. We got approved for our request. Please see attached amendment of modification contract.

We will follow to our plans, approaches, and goals that I specified for each aim in the original proposal.

Aim 1: Investigate the inflammatory profile of canonical and hybrid TANs, (timeline 1-12 months). This aim is completed.

Aim 2: Investigate the effects of canonical and hybrid TANs on T cell responses and on the maturation and function of dendritic cells, (timeline 1-18 months). This aim is completed

Aim 3: Define the cytotoxic phenotype of canonical and hybrid neutrophils and the mechanisms by which these neutrophils inhibit tumor growth, (timeline 12-24 months).

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Although we have partially completed this aim, there are remaining experiments that we will perform experiments according to our original plan described in the proposal. Having optimized our flow cytometry based approach to study the potential mechanisms by which different neutrophil populations might mediate Ab-dependent cellular cytotoxicity against tumor cells, we will determine the contribution of neutrophil subsets to Ab-dependent cellular cytotoxicity. Tumoricidal activity of neutrophils may depend on the size of tumor cell and the level of expression of surface tumor antigen. To address these points in our model, we will use large size with high level of expression of $EGFR^{hi}$ A431 tumor cell line and large size with low level of expression of $EGFR^{lo}$ A549 tumor cell line. These tumor cell lines will be opsonized with anti-EGFR monoclonal humanized antibodies (cetuximab) that are actively used in clinic. Also, we will use small size Daudi tumor cell line opsonized with anti-CD20 Abs (rituximab). For these experiments we will mix canonical and hybrid neutrophils with large size tumor cells (A549) opsonized with cetuximab (anti-EGFR Abs) or with small size tumor cells (Daudi) opsonized with retuximab (anti-CD20 Abs). We will determine whether the canonical and hybrid neutrophils able to mediate ADCC or ADP as described above. $Fc_{\gamma}R$-bearing monocyte-derived macrophages and blood NK cells will be used in this assay as a positive control for ADP and ADCC, respectively.

Once we have established the mechanisms of hybrid neutrophil cytotoxicity triggered by tumor-specific Abs in vitro, we will start to explore the clinical potential of hybrid cells generated from BM to mediate ADCC in vivo. We will inject 5 million human neutrophils intratumorally into established human lung cancer cell line-derived tumors ($100mm^3$A549 lung cancer xenografts) growing in NSG mice and subsequently measure tumor volume. In addition, we will characterize the ability of different neutrophil subsets to induce ADCC using the anti-EGFR mAb cetuximab.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?
To date, the characterization of the human lung tumor microenvironment is still in its infancy and the functional cross-talk between immune and tumor cells in humans remains largely unexplored. A better understanding of the interaction between cancer cells and the lung microenvironment may allow tumor immunologists to develop novel strategies to improve anti-tumor immune responses. Tumor-recruited myeloid cells represent a significant portion of inflammatory cells in the tumor microenvironment and influence nearly all steps of tumor progression. Among the different types of myeloid cells, tumor-associated macrophages (TAMs) have been the best characterized and are generally considered pro-tumoral in murine tumor models. The role of tumor-associated neutrophils (TANs) in cancer progression remains unclear and has only been recently investigated in murine models. Characterization of human TANs is even less well-developed. Numerous findings in murine model systems suggest a predominantly pro-tumoral role for neutrophils in cancer development. However, there are crucial species differences in the evolution of tumors, genetic diversity, immune and inflammatory response, and intrinsic biology of neutrophils that we postulate have a profound impact on tumor development and the function of neutrophils in mouse tumors versus human. A crucial difference is that the majority of mouse tumor models lack the prolonged initial phases of multistage tumor evolution present in humans, such as elimination and equilibrium phases, where anti-tumoral mechanisms are activated. It is a sobering fact that the majority of cancer immune therapies that work well in mice fail to provide similar efficacy in humans; the average rate of successful translation from animal models to clinical cancer trials is less than 8% (2). Thus, there is a continuing need to develop new and innovative approaches to characterize granulocytes in human cancers and to characterize the variety of their functions in the human tumor microenvironment.

Our study generates new knowledge about human TANs and exerts a sustained influence on the field. Therefore, this work is a first-of-its kind and will important ramifications for patients with lung cancer. Specifically, we for the first time provided the detailed phenotypic and functional characteristics TAN and their subsets in human early-stage cancer. We provide the first evidence of two subsets of TANs in human lung cancer. All TANs had an activated phenotype and could support (rather than inhibit) T cell functions to some degree. In contrast to mouse tumor models, our data demonstrate that in patients with early stage lung cancer, TANs do not significantly contribute to the inhibition of T cell responses. In fact, the TANs isolated from a vast majority of small size early stages tumors were actually able to stimulate T cell response to varying degrees. We identified a subset of TAN in early-stage lung tumors that can undergo a unique differentiation process resulting in formation of specialized subset of APC-like hybrid neutrophils. These hybrid TANs had enhanced ability to trigger and support anti-tumor T cell responses in direct cell-cell interactions. This property of hybrid neutrophils may provide new opportunities to boost the efficacy of vaccines based on cytotoxic T lymphocyte induction. Understanding the role of TANs in regulating T cell responses is particularly important because cytotoxic T lymphocytes are the primary effector cells mediating antigen-driven anti-tumor immunity.

These results have been published in JCI, Cancer Cell and Clinical Cancer Research. In addition these results have been presented at a major cancer immunology conferences organized by AACR and AAI (please see details in PRODUCTS). This knowledge will allow us to develop different therapeutic strategies to regulate the function of TANs depending on tumor stage in human. Understanding how to direct and maintain the human TANs towards anti-tumor effector cells will open new therapeutic options in the future design of active immunotherapy to potentially boost natural or vaccine-induced anti-tumor immunity.

What was the impact on other disciplines?
Nothing to Report
What was the impact on technology transfer?
Nothing to Report
What was the impact on society beyond science and technology?
Nothing to Report

CHANGES/PROBLEMS

Changes in approach and reasons for change: Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them.

We requested 1-year no-cost extension because we were delayed in the initial onset of the project for IRB related issues. We will be completing the proposed Aims with no modifications. Please see the attached letter.

We got approved for our request. Please see attached amendment of modification contract.
The purpose of this modification is to extend the period of performance by 12 months at no additional cost to the Government, per the recipient's request received on 24 August 2017.The annual report will be due no later than 29 October 2017. The final technical report is due no later than 29 December 2018.

Changes that had a significant impact on expenditures. Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Nothing to Report

Significant changes in use or care of human subjects: Nothing to Report

Significant changes in use or care of vertebrate animals: Nothing to Report

Significant changes in use of biohazards and/or select agents: Nothing to Report

PRODUCTS

Publications, conference papers, and presentations

Journal publications:

2016

1. **Eruslanov E** (corresponding author), Bhojnagarwala P, Quatromoni J, Stephen T, Ranganathan A, Deshpande C, Akimova T, Vachani A, Litzky L, Hancock W, Conejo-Garcia J, Feldman M, Albelda S, and Singhal S. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. **The Journal of Clinical Investigation**, 2014, Dec 1; 124(12): 5466-80, highlighted as a featured paper.
2. Singhal S, Bhojnagarwala P, O'Brien S, Moon E, Garfall A, Rao A,. Quatromoni J, Stephen T, Litzky L, Deshpande D, Feldman M, Hancock W, Conejo-Garcia J, Albelda S, and **Eruslanov E.** Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen Presenting Cell Features in Early-Stage Human Lung Cancer. **Cancer Cell**, 2016, Jul 11;30(1):120-35. doi: 10.1016/j.ccell.2016.06.001
3. Moon E, Ranganathan R, **Eruslanov E**, Kim S, Newick K, O'Brien S, Lo A, Liu X, Zhao Y, and Albelda S. Blockade of Programmed Death 1 Augments the Ability of Human T cells Engineered to Target NY-ESO-1 to Control Tumor Growth after Adoptive Transfer. **Clinical Cancer Research**; 2016 Jan 15;22(2):436-47. doi: 10.1158/1078-0432.CCR-15-1070.
4. T Condamine, G. Dominguez, Je-In Youn, A. Kossenkov, S Mony, KAlicea-Torres, E Tcyganov, A Hashimoto, Y Nefedova, C Lin, S Partlova, A Garfall, D. Vogl, X Xu, S Knight, G Malietzis, G Han Lee, **E Eruslanov**, S Albelda, X Wang, J Mehta, M Bewtra, A Rustgi, N Hockstein, R Witt, G Masters, B Nam, D Smirnov, M Sepulveda and D Gabrilovich. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. **Science Immunology**. 5 August 2016, DOI: 10.1126/sciimmunol.aaf8943

2017

1. **Eruslanov E,** Singhal S, and Albelda S. Mouse versus Human Neutrophils in Cancer: A Major Knowledge Gap. ***Trends in Cancer***. 2017, Vol. 3, No. 2, p149-160
2. **Eruslanov E.** Phenotype and function of tumor-associated neutrophils and their subsets in early-stage human lung cancer. ***Cancer Immunology, Immunotherapy***. 2017, Aug; 66(8): p997-1006
3. Svoronos N, Perales-Puchalt A, Allegrezza MJ, Rutkowski MR, Payne KK, Tesone AJ, Nguyen JM, Curiel TJ, Cadungog MG, Singhal S, **Eruslanov E**, Zhang P, Tchou J, Zhang R, Conejo-Garcia JR. Tumor Cell-Independent Estrogen Signaling Drives Disease Progression through Mobilization of Myeloid-Derived Supressor Cells. ***Cancer Discovery***. 2017 Jan;7(1):p72-85.
4. Stephen TL, Payne KK,Chaurio RA, Allegrezza MJ, Zhu H, Perez-Sanz J, Perales-Puchalt A, Nguyen JM, Vara-Ailor AE, **Eruslanov E**, Borowsky ME, Zhang R, Laufer TM, Conejo-Garcia JR. SATB1 Expression Governs Epigenetic Repression of PD-1 in Tumor-Reactive T Cells. ***Immunity.*** 2017, Volume 46, Issue 1, 17, p51–64
5. Akimova T, Zhang T, Negorev D, Singhal S, Stadanlick J, Rao A, Annunziata M, Levine M, Beier U, Diamond J, Christie J, Albelda S, **Eruslanov E**, and Hancock W. Tumor Treg upregulate a quartet of "Treg-locking" transcription factors. ***JCI Insight,*** 2017; 2(16): e94075.
6. Bruno T, Ebner P, Moore B, Squalls O, Waugh K, **Eruslanov E**, Singhal S, Mitchell J, Franklin W, Merrick D, McCarter M, Palmer B, Kern J, and Jill Slansky. Antigen-presenting tumor B cells affect CD4+TIL phenotype in non-small cell lung cancer patients. ***Cancer Immunology Research***, August 28, 2017, DOI: 10.1158/2326-6066.CIR-17-0075

Books or other non-periodical, one-time publications.
Nothing to Report

Other publications, conference papers, and presentations.

The results were presented at the following meetings:

2017

- AACR Annual Meeting 2017**,** Walter E. Washington Convention Center, April 1-5, Washington, D.C. **poster presentation**
- AAI Annual Meeting, Immunology 2017, May 12-16, Washington, D.C. Oral presentation on the Major Forum. **Invited Speaker**
- The Division of Transplant Immunobiology and the Department of Pathology and Laboratory Medicine at the Children's Hospital of Philadelphia. Philadelphia, September 20th, 2017, **Invited Speaker**
- Immuno-oncology seminar, Translational Genomics Research Institute, Phoenix, April 25-26. **Invited Speaker**
- Immuno-oncology seminar, AstraZeneca, Waltham, MA, 2017, March 16, **Invited Speaker**

2016

- The Society For Leukocyte Biology's 49th Annual Meeting and "Neutrophil 2016": Neutrophils and Other Leukocytes, University of Verona, Verona, Italy, September 15-17, 2016, **Invited Speaker**.
- Regulatory Myeloid Suppressor Cells Conference, Philadelphia, The Wistar Institute, June 16-19, 2016 **Invited Speaker**.
- Immunology School "Regulation of Lung Inflammation", Moscow, Russia, May11-13, 2016. **Invited Speaker**.
- AACR annual meeting: The function of tumor microenvironment in cancer. San Diego, Jan 7, 2016, **poster presentation.**

Conference presentations:

1. AACR Annual Meeting 2017**,** Walter E. Washington Convention Center, April 1-5, Washington, D.C. APC-like Tumor-Associated Neutrophils in Human Lung Cancer. **poster presentation**
2. E. Eruslanov. Tumor-associated neutrophils with antigen-presenting cell features in human lung cancer. American Association of Immunologists (AAI) annual meeting Immunology 2017, Major Symposium F: Neutrophils Function in Autoimmunity, Infection, and Cancer. May 12-16, Washington, D.C. Invited Speaker
3. **E. Eruslanov**. Tumor-Associated Neutrophils in Human Lung Cancer. Inflammation, Immunity and Cancer: The Society For Leukocyte Biology's 49th Annual Meeting and "Neutrophil 2016": Neutrophils and Other Leukocytes, University of Verona, Verona, Italy, September 15-17, 2016, Invited Speaker.*****
4. **E. Eruslanov**, Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen Presenting Cell Features in Early-Stage Human Lung Cancer. Regulatory Myeloid Suppressor Cells Conference, Philadelphia, The Wistar Institute, June 16-19, 2016 Invited Speaker.*****

5. **E. Eruslanov,** Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen Presenting Cell Features (Hybrid TANs) in Early-Stage Human Lung Cancer. Immunology School "Regulation of Lung Inflammation", Moscow, Russia, May11-13, 2016. Invited Speaker.*****
6. **E. Eruslanov**, P Bhojnagarwala, J Quatromoni, S O'Brien, E Moon, T Stephen, A Rao, A Garfall, W Hancock, J Conejo-Garcia, C Deshpande, M Feldman, S Singhal and S Albelda. The origin and role of APC-like hybrid tumor-associated neutrophils in early-stage human lung cancer. AACR annual meeting: The function of tumor microenvironment in cancer. San Diego, Jan 7, 2016, poster presentation*****

(*) presentation produced a manuscript.

Website(s) or other Internet site(s)
Penn Medicine News Site:
- *http://www.uphs.upenn.edu/news/News_Releases/2016/07/eruslanov/*

Technologies or techniques
Nothing to Report

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Evgeniy Eruslanov
Project Role:	PI
Researcher Identifier Penn ID	14731836
Nearest person month worked:	5
Contribution to Project:	Dr. Eruslanov has performed some experiments, and is responsible for supervising the overall conduct of the project, planning and coordinating experiments, analyzing and interpreting data, and writing results and progress reports.
Funding Support:	DoD LC140199

Name:	Michael Annunziata
Project Role:	Research Specialist
Researcher Identifier Penn ID	54294537
Nearest person month worked:	6
Contribution to Project:	Michael obtained and prepared the tumor tissues for analysis. He has also performed flow cytometry, cell culturing and functional assays.
Funding Support:	DoD LC140199

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

"Nothing to Report."

What other organizations were involved as partners?

"Nothing to Report."

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

"Nothing to Report."

QUAD CHARTS:

"Nothing to Report."

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APPENDICES

Please cite this article in press as: Singhal et al., Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen-Presenting Cell Features in Early-Stage Human Lung Cancer, Cancer Cell (2016), http://dx.doi.org/10.1016/j.ccell.2016.06.001

CellPress

Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen-Presenting Cell Features in Early-Stage Human Lung Cancer

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http://dx.doi.org/10.1016/j.ccell.2016.06.001

SUMMARY

Based on studies in mouse tumor models, granulocytes appear to play a tumor-promoting role. However, there are limited data about the phenotype and function of tumor-associated neutrophils (TANs) in humans. Here, we identify a subset of TANs that exhibited characteristics of both neutrophils and antigen-presenting cells (APCs) in early-stage human lung cancer. These APC-like "hybrid neutrophils," which originate from $CD11b^{+}CD15^{hi}CD10^{-}CD16^{low}$ immature progenitors, are able to cross-present antigens, as well as trigger and augment anti-tumor T cell responses. Interferon-γ and granulocyte-macrophage colony-stimulating factor are requisite factors in the tumor that, working through the Ikaros transcription factor, synergistically exert their APC-promoting effects on the progenitors. Overall, these data demonstrate the existence of a specialized TAN subset with anti-tumor capabilities in human cancer.

INTRODUCTION

Tumor-associated inflammation contributes to cancer development and progression and is often associated with a high degree of inflammatory cell infiltration (Grivennikov et al., 2010). Tumor-associated neutrophils (TANs) represent a significant portion of tumor-infiltrating cells and accumulate in many types of cancers, including lung cancer (Carus et al., 2013; Ilie et al., 2012). Although the role of TANs in tumor development is beginning to be investigated in murine models, it remains largely unexplored in humans.

In murine studies, it appears that TANs can exert both pro-tumor and anti-tumor effects (Brandau, 2013; Fridlender et al., 2009). Numerous studies have shown that neutrophils can promote tumor progression by degrading matrix, immunosculpting, stimulating tumor cell proliferation, increasing metastasis, and enhancing angiogenesis (Houghton, 2010; Piccard et al., 2012). However, they can also exert anti-tumor functions such as inducing tumor cell death via their powerful antimicrobial killing machinery (Dallegri and Ottonello, 1992; van Egmond and Bakema, 2013) and by producing factors to recruit and activate cells of the innate and adaptive immune system (Mantovani et al., 2011). Given these varying effects of mouse TANs on tumor growth, the paradigm of anti-tumor "N1 neutrophils" versus pro-tumor "N2 neutrophils" was proposed (Fridlender et al.,

Significance

Tumor-associated neutrophils (TANs) represent a significant fraction of the inflammatory cells in the tumor microenvironment; however, the contribution of these cells in inhibiting or promoting tumor expansion in humans remains unclear. Although the concept of neutrophil phenotypic and functional diversity has emerged in murine tumor models, it is unknown whether TAN subsets with different functions exist in humans. Here, we provide evidence that early-stage lung tumors can induce the formation of a unique subset of TANs that can trigger and support anti-tumor T cell responses. These findings demonstrate the potential anti-tumor role of TANs in early-stage cancer and may provide opportunities to boost the anti-tumor efficacy of cytotoxic T lymphocytes.

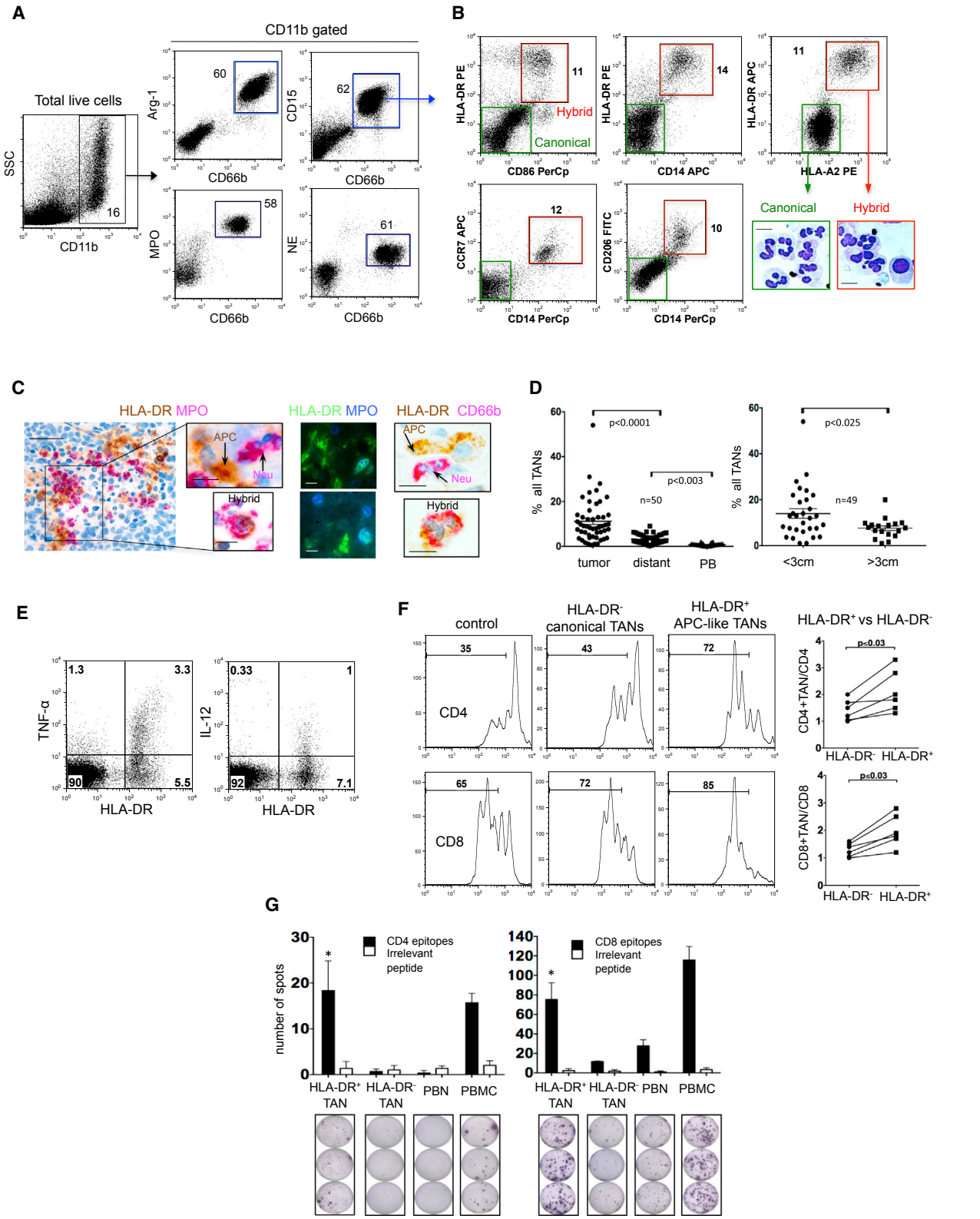

Figure 1. A Subset of TANs with Hybrid Characteristics of Neutrophils and APCs

(A) A single-cell suspension was obtained from fresh tumor and the expression of the indicated granulocytic markers was analyzed by flow cytometry on gated live CD11b cells. Total TANs are shown in blue boxes.

(legend continued on next page)

2009). However, most of these data were derived from mouse models that use tumor cell lines adapted to grow rapidly in vivo and have thus already undergone cancer immunoediting (Schreiber et al., 2011). These models are also characterized by high tumor burden, minimal matrix, and rapid tumor growth. Because these features are dissimilar to human cancers that evolve slowly over time, the role of tumor-infiltrating myeloid cells in human cancers may not be the same and the function of human TANs, particularly in the early stages of tumor development, remains largely unexplored.

Understanding the role of TANs in the regulation of the T cell response in cancer patients is important because the cytotoxic T lymphocytes are the major effector cells mediating antigen-driven anti-tumor immunity. We recently demonstrated that early-stage lung cancers are highly infiltrated with activated neutrophils and that these TANs exhibit heterogeneous expression of T cell co-stimulatory molecules (Eruslanov et al., 2014). In contrast to the data from murine studies, TANs isolated from vast majority of small early-stage tumors were not immunosuppressive, but in fact stimulated T cell responses (Eruslanov et al., 2014). Interestingly, the T cell activation property of TANs became less prominent with disease progression, consistent with the emerging concept of an immunogenic "switch" from anti-tumor to pro-tumor phenotype (Granot and Fridlender, 2015).

As part of our phenotypic analysis of early-stage lung cancer TANs (Eruslanov et al., 2014), we identified a subset of cells exhibiting the hybrid phenotype of both neutrophils and antigen-presenting cells (APCs). We hypothesized that early-stage tumors, where the immunosuppressive environment might not be fully developed, can drive recruited granulocytes to further differentiate into a specialized cell subset with strong T cell stimulatory activity. The purpose of this study was to characterize the phenotype, function, and origin of these hybrid cells in lung cancer patients.

RESULTS

Early-Stage Human Lung Cancers Accumulate a Neutrophil Subset with a Composite Phenotype of Granulocytes and APCs

Since TANs in patients with early-stage lung cancer have the ability to heterogeneously express some T cell co-stimulatory molecules (Eruslanov et al., 2014), we postulated that there might be a subset of TANs with characteristics of APCs. We thus analyzed the expression of APC surface markers on neutrophils from three locations: lung cancer tissue, adjacent (within the same lobe) lung parenchyma (termed "distant tissue"), and peripheral blood (Figure S1A). We performed phenotypic analysis of 50 random patients with stage I–II non-small cell lung cancer (NSCLC). Detailed characteristics of all patients involved in this study are shown in Table S1. Fresh tissue was digested using defined conditions that minimize enzyme-induced ex vivo effects on the viability, premature activation, phenotype, and function of neutrophils (Quatromoni et al., 2015). Previously, we performed extensive phenotypic analysis of neutrophils in NSCLC and characterized TANs as $CD11b^{+}CD15^{hi}CD66b^{+}MPO^{+}Arg1^{+}CD16^{int}IL\text{-}5R\alpha^{-}$ cells (Eruslanov et al., 2014). Importantly, all $CD66b^{+}CD11b^{+}$ cells also expressed the other neutrophil/myeloid cell markers CD15, MPO (myeloperoxidase), Arg-1 (arginase-1), and NE (neutrophil elastase) at very high levels (Figure 1A, blue boxes) and thus could be segregated from other $CD15^{lo}MPO^{lo}NE^{lo}Arg\text{-}1^{-}$ non-granulocytic $CD11b^{+}$ myeloid cells. Since there was a high concordance among the selected neutrophil markers, for our studies we defined TANs as $CD15^{hi}CD66b^{+}CD11b^{+}$ cells. Our analysis revealed that the majority of neutrophils from lung tumors, which we term "canonical TANs," expressed only these classic neutrophil markers (Figures 1A and S1A). However, we also identified TANs with surface expression of additional markers normally expressed on APCs, specifically human leukocyte antigen (HLA)-DR, CD14, CD206, CD86, and CCR7 (Figures S1B–S1F). These receptors were completely absent in peripheral blood neutrophils (PBNs). The "distant tissue" neutrophils also expressed these APC markers, albeit at much lower levels in comparison with TANs.

Further analysis revealed that the APC markers ($CD14^{+}HLA\text{-}DR^{+}HLA\text{-}ABC^{hi}CCR7^{+}CD86^{+}CD206^{+}$) were co-expressed on a unique subset of $CD11b^{+}CD66b^{+}CD15^{hi}$ TANs (Figure 1B), exhibiting a composite phenotype of canonical neutrophils and APCs. We termed this subset "APC-like hybrid TANs." This population of hybrid TANs expressed some markers of the APC phenotype (e.g., CD14, HLA-DR, CCR7, CD86, and CD206) but lacked other defining markers of "professional APC" such as CD209, CD204, CD83, CD163, CD1c, and CCR6 (data not shown). Of note, the expression of CD206, CCR7, and CD86 varied, whereas there was a consistent co-expression of HLA-DR and CD14 on hybrid TANs. Cytospins prepared from flow-sorted $HLA\text{-}DR^{-}$ canonical and $HLA\text{-}DR^{+}$ hybrid TANs revealed that some of the hybrid TANs had round and oval nuclear shapes in comparison with the classic nuclear segmentation of canonical TANs (Figure 1B). Histological review of lung tumors also revealed "double-positive" $MPO^{+}HLA\text{-}DR^{+}$ and $CD66b^{+}HLA\text{-}DR^{+}$ TANs that were scattered throughout lung tumors

(B) Flow cytometric analysis of the expression of APC markers on gated $CD11b^{+}CD15^{hi}CD66b^{+}$ TANs. The representative cytomorphology of canonical (green boxes) and APC-like hybrid TANs (red boxes) in NSCLC. Scale bar, 10 μm.

(C) The presence of APC-like hybrid TANs in tumor detected by immunohistochemistry and immunofluorescence double staining. Scale bar, 50 μm (left image) and 10 μm (other images).

(D) The frequency of APC-like hybrid neutrophils in tumors, distant lung tissue, and peripheral blood (PB) (right graph) and in tumors of different sizes (left graph) (line represents mean ± SEM, n = 50, one-way ANOVA test and unpaired t test). APC-like hybrid TANs were defined as live $HLA\text{-}DR^{+}CD11b^{+}CD15^{hi}CD66b^{+}$ cells.

(E) Intracellular TNF-α and IL-12 production by $HLA\text{-}DR^{+}$ hybrid or $HLA\text{-}DR^{-}$ canonical TANs after stimulation with LPS. TANs were gated on $CD11b^{+}CD15^{hi}CD66b^{+}$ cells. Representative results from one of five experiments are shown.

(F) The proliferation of autologous CFSE-labeled PBMC stimulated with plate-bound anti-CD3 Abs in the presence of hybrid $HLA\text{-}DR^{+}$ or canonical $HLA\text{-}DR^{-}$ TANs. T cell stimulatory activity was defined as the ratio $CFSE^{lo}$ (T cells + TANs)/$CFSE^{lo}$ (T cells) (n = 6, Wilcoxon matched-pairs rank test).

(G) Autologous virus-specific memory T cell responses in the presence of APC-like hybrid $HLA\text{-}DR^{+}$ or canonical $HLA\text{-}DR^{-}$ TANs. IFN-γ-ELISPOT assay (mean ± SEM, n = 3, *$p \leq 0.01$ canonical versus hybrid, Mann-Whitney test).

See also Figure S1.

(Figure 1C). Additionally, we detected a small, but clearly distinguishable population of HLA-DR$^+$CD15^{hi}CD66b$^+$CD11b$^+$ cells in the draining lymph nodes of several lung cancer patients (Figure S1G).

The frequency of this identified subset of TANs varied from 0.5% to 25% among all TANs (Figure 1D) and from 0.1% to 4.3% among all cells in tumor digests (Figure S1H). The hybrid population was significantly higher in patients with adenocarcinoma compared with patients with squamous cell carcinoma (Figure S1I). There were no significant associations between the frequency of APC-like TANs and tumor stage or smoking history (Figures S1J and S1K). Interestingly, we found a significantly smaller percentage of HLA-DR$^+$ hybrid neutrophils among TANs in large tumors (diameter >3 cm) versus the small tumors (diameter <3 cm) (Figures 1D and S1L). Thus, the hybrid population appears to decline as tumors enlarge, and is completely absent in tumors greater than 5–7 cm in diameter. Together, these data demonstrate that neutrophils in some early-stage lung tumors undergo unique phenotypic changes, yielding a subset of TANs with composite characteristics of neutrophils and APC.

APC-like Hybrid TANs Stimulate and Support T Cell Responses

Previously, we showed that TANs isolated from small, early-stage lung tumors were able to stimulate antigen non-specific T cell responses (Eruslanov et al., 2014). Having identified these APC-like TANs, we hypothesized that this subset may be primarily responsible for stimulating T cell responses in these early-stage lung tumors.

We first evaluated the functional activity of APC-like TANs to ensure that these activated cells were not "exhausted" or hypofunctional. TANs were thus isolated from tumors and stimulated with lipopolysaccharide (LPS). After LPS stimulation, HLA-DR$^+$ hybrid TANs produced much more tumor necrosis factor α (TNF-α) and interleukin-12 (IL-12) when compared with HLA-DR$^-$ canonical TANs (Figure 1E). Furthermore, HLA-DR$^+$ hybrid TANs phagocytosed *Escherichia coli* bioparticles more efficiently than HLA-DR$^-$ canonical TANs (Figure S1M). These data demonstrate that APC-like hybrid TANs are fully functional and, in fact, perform major functions such as cytokine production and phagocytosis superior to canonical TANs.

To determine the effect of APC-like hybrid TANs on T cell responses, we isolated TAN subsets by flow cytometry cell sorting (Figures S1N and S1O). Each sorted TAN subset was co-cultured with autologous carboxyfluorescein succinimidyl ester (CFSE)-labeled peripheral blood mononuclear cells (PBMCs) that had been stimulated with plate-bound anti-CD3 antibodies (Abs) (Figure 1F). We observed that the proliferation of CD4 and CD8 cells after 4 days of stimulation was markedly augmented after exposure to HLA-DR$^+$ hybrid TANs versus the HLA-DR$^-$ canonical TANs (Figure 1F).

We next determined whether APC-like hybrid TANs could trigger and sustain antigen-specific T cell responses. Therefore, we co-cultured autologous T cells with TAN subsets that had been pulsed with mixtures of overlapping peptides from commercially available peptide pools. Each peptide pool corresponded to defined HLA class I or II restricted T cell epitopes from cytomegalovirus, Epstein-Barr virus, influenza virus, or *Clostridium tetani* designed to stimulate T cells with a broad array of HLA types. As shown in Figure 1G, the HLA-DR$^+$ hybrid TANs efficiently triggered memory CD8 and CD4 T cell responses to HLA class I and II restricted T cell epitopes, respectively. Canonical TANs and PBNs induced only weak CD8 T cell responses and did not trigger CD4 T cell responses. Together, these data demonstrate that HLA-DR$^+$ hybrid TANs are able to function as efficient APCs.

Long-Lived Immature Neutrophils Recapitulate the Phenotype of APC-like Hybrid TANs in the Presence of Tumor-Derived Factors

Given the potential anti-tumor activity of APC-like TANs due to their strong stimulatory effect on T cell responses, we investigated the mechanisms by which these cells could originate and expand in the human tumor microenvironment.

We collected tumor-conditioned media (TCM) from digested lung cancers that contained $\geq$15% of hybrid TANs among all TANs (termed hybrid-inducing TCM). We exposed purified PBNs to hybrid-inducing TCM and found that PBNs did not differentiate into the HLA-DR$^+$CD14$^+$ neutrophils (data not shown) and died within 3 days (Figure 2A). To determine whether more immature neutrophils with a higher degree of plasticity differentiate into APC-like hybrid neutrophils, we obtained a highly enriched population of immature human bone marrow neutrophils (BMNs). Isolated BMNs expressed the myeloid/granulocytic specific markers CD11b, CD66b, CD15, Arg-1, NE, and MPO and were mostly "band"-like immature neutrophils in appearance (Figures 2B and S2A). Of note, the purified BMNs did not express HLA-DR and CD14 and were not contaminated with macrophages and monocytes (Figure S2A). Unlike blood neutrophils, about 40% of these BMNs could survive in cell culture for up to 1 week and their viability was dramatically increased in the presence of TCM (Figures 2A, 2C, and S2B). Thus, BMNs with a prolonged lifespan in vitro provided us with large quantities of cells that could be used to model the origins and differentiation process of neutrophils in the tumor microenvironment.

After 7 days of incubation of BMNs with hybrid-inducing TCMs, we observed the formation of a cell subset that retained all its granulocytic markers (Figures 2B and 2D) and acquired the same phenotype as the tumor-derived hybrid TANs (HLA-DR$^+$CD14$^+$CD86$^+$CD206$^+$CCR7$^+$) (Figure 2E). Similar to hybrid TANs, most of the BMNs also changed their nuclear shape from band-like to oval when they converted into hybrid BMNs (Figure 2B). A detailed phenotypic comparison of PBNs, BMNs, and bone marrow (BM)- and tumor-derived hybrid neutrophils is summarized in Figure 2D. The differentiation of BMNs into HLA-DR$^+$CD14$^+$ APC-like hybrid BMNs after exposure to hybrid-inducing TCM was donor dependent and varied from 20% to 80% of the initial BMN population (Figure S2C). BMNs began to upregulate CD14 within 24 hr of co-culturing with hybrid-inducing TCM, while the expression of HLA-DR, CD86, CCR7, and CD206 markers did not appear until day 4 (Figure S2D). This suggests that these late APC markers are synthesized de novo.

Similar to hybrid TANs, differentiated hybrid BMNs acquired only the partial phenotype of dendritic cells (DC) and macrophages (Mph) (HLA-DR$^+$CD14$^+$CD86$^+$CD206$^+$) (Figures 2E–2G). The hybrid subset of BMNs and TANs differed from BM-derived DC and Mph by absence of CD1c, CD83, CD163, and CD209

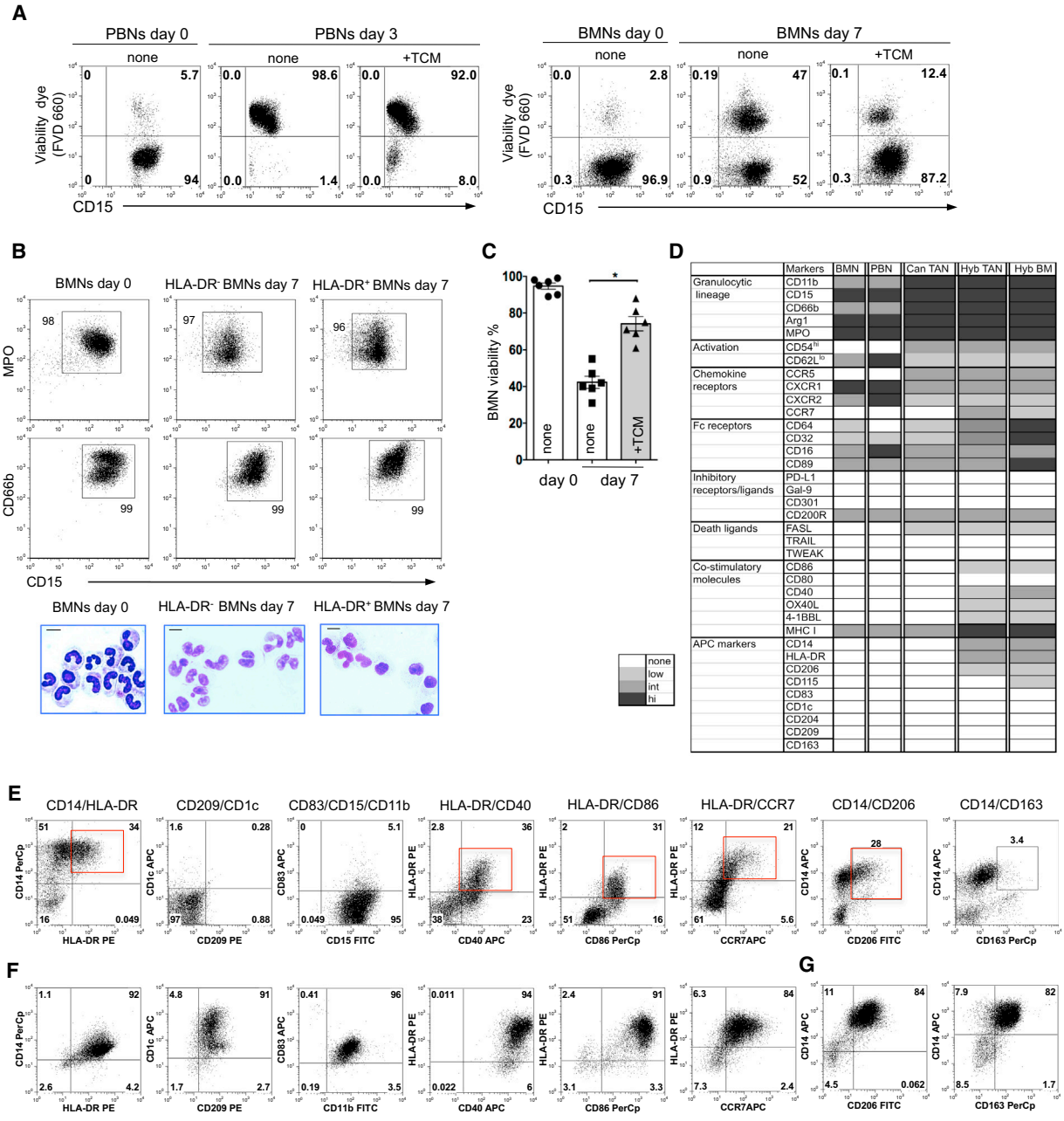

Figure 2. Tumor-Derived Factors Differentiate Long-Lived Immature BMNs into a Hybrid Subset with a Partial Phenotype of Dendritic Cells and Macrophages

(A) Fixable viability dye eFluor 660 (FVD 660) was used to discriminate viable neutrophils in cell culture. Representative dot plots from one of six experiments are shown.

(B) Flow cytometric analysis of the expression of MPO, CD66b, and CD15 markers on freshly isolated BMNs (day 0) and BMNs cultured with (HLA-DR$^+$ BMNs) or without hybrid-inducing TCM (HLA-DR$^-$ BMNs) for 7 days. Cytospins show the cytomorphology of these BMNs. Scale bar, 10 μm.

(C) Survival of BMNs in the cell culture in the presence or absence of TCM. Viability dye FVD 660 was used to discriminate viable BMNs in cell culture (mean ± SEM, n = 6, *p ≤ 0.01, Wilcoxon matched-pairs rank test).

(D) Heatmap comparing the phenotypes of BMNs, PBNs, canonical TANs (Can TAN), hybrid TANs (Hyb TAN), and BM-derived hybrid neutrophils (Hyb BM).

(E–G) Flow cytometric analysis of the expression of indicated APC markers on BM-derived hybrid neutrophils (E) (red boxes), dendritic cells (F), and macrophages (G). Expression of APC markers was analyzed by flow cytometry on gated CD11b$^+$CD15^{hi}CD66b$^+$ BMNs.

See also Figure S2.

markers, and low expression of CD40, CD86, CD115, and CCR7 (Figures 2D–2G). The level of the transcription factor IRF8, which regulates monocyte/DC lineage commitment (Yanez et al., 2015), was not dramatically changed in hybrid BMNs and was much lower than the amount detected in BM-derived Mph and DC (Figure S2E).

We next asked whether the differentiated APC-like hybrid BMNs could proliferate in the presence of hybrid-inducing TCM and thus represent a self-maintained population of neutrophils. A bromodeoxyuridine (BrdU) incorporation assay revealed that within 24 hr of treatment with hybrid-inducing TCM, 10%–15% of BMNs begin to synthesize DNA in vitro (Figure S2F). As the differentiation process progressed, a small proportion of HLA-DR$^-$ BMNs continued to incorporate BrdU up to day 8, whereas the differentiated HLA-DR$^+$ neutrophils lost proliferative potential (Figure S2F).

Given that the frequency of hybrid TANs was reduced in large tumors (Figure 1D), we hypothesized that hypoxia, which is strongly associated with the tumor progression, may negatively regulate the formation of hybrid neutrophils. Thus, BMNs were cultured in the presence of hybrid-inducing TCM for 6 days under normoxic (5% CO_2 and 21% O_2) and hypoxic (5% CO_2 and 5% O_2) cell culture conditions. We also cultured BMNs in the presence of hybrid-inducing TCM and cobalt chloride, an agent that induces hypoxia-inducible factor 1α (HIF-1α), the main transcriptional factor activated in hypoxic conditions (Dai et al., 2012). We found that the development of hybrid CD14$^+$HLA-DR$^+$ neutrophils was profoundly inhibited under these hypoxic and hypoxia-simulating conditions (Figure 3A).

IFN-γ and GM-CSF Are Requisite Factors in the Tumor Microenvironment for the Development of Hybrid Neutrophils

To determine the particular tumor-specific factors that promote the formation of hybrid TANs, we screened primary TCMs collected from 20 consecutive lung cancer patients, and categorized the TCMs based on their ability to induce: (1) the full phenotype of hybrid cells (CD14$^+$HLA-DR$^+$CD11b$^+$CD66b$^+$CD15^{hi}) (Figure 3B, example TCM #41); (2) the partial phenotype of hybrid cells (CD14$^+$HLA-DR$^-$CD11b$^+$CD66b$^+$CD15^{hi}) (Figure 3B, example TCM #63); or (3) no phenotypic changes (Figure 3B, example TCM #58). We evaluated each TCM using a multiplex cytokine/chemokine bead assay and found that those TCMs that induced CD14$^+$HLA-DR$^+$ hybrid cells had increased amounts of granulocyte-colony stimulating factor (G-CSF), IL-6, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), macrophage inflammatory protein-1α (MIP-1α), TNF-α, monocyte chemoattractant protein-1 (MCP-1), and monokine induced by IFN-γ (MIG) compared with TCMs that did not induce hybrid cells. When we tested the ability of each of these factors (at the low concentrations found in the TCMs) to induce the CD14$^+$HLA-DR$^+$ hybrid phenotype in BMNs, we found that only IFN-γ and GM-CSF were able to induce the phenotype, although in a relatively low percentage of cells (Figures 3B and S3A). However, we observed that these factors worked in a synergistic manner: when combined at very low concentrations of 50 pg/ml of each factor, they induced expression of APC markers in a large proportion (>40%) of the cells in a donor-dependent fashion (Figures 3B and S3A). The addition of neutralizing monoclonal antibodies for either IFN-γ or GM-CSF completely inhibited the formation of BM hybrid cells in the presence of hybrid-inducing TCM (Figure 3C), thereby confirming that both IFN-γ and GM-CSF play a key role in the induction process. Interestingly, incubation of BMNs with a low dose of GM-CSF (50 pg/ml) and increasing concentrations of IFN-γ (from 50 pg/ml to 20 ng/ml) resulted in the expansion of CD14$^+$HLA-DR$^+$ BMNs from 40% to 96% among all BMNs (Figure 3D, upper panel). However, the treatment of BMNs with IFN-γ at a concentration of more than 1 ng/ml gradually induced the expression of PD-L1 on the HLA-DR$^+$ BMNs (Figure 3D, lower panel), resulting in the formation of hybrid neutrophils with T cell suppressive activity (described in detail below).

We next analyzed the frequency of APC-like TANs in the tumor digests, and, in parallel, measured the concentration of IFN-γ and GM-CSF in the supernatants collected from digested autologous tumor cell cultures. Figures 3E and 3F demonstrate that the levels of IFN-γ and GM-CSF were statistically higher in tumors where there was a high proportion of hybrid TANs (>10% of all TANs). However, the generation of hybrid neutrophils in vivo is most likely more complex and not solely due to IFN-γ and GM-CSF levels, because the absolute levels of IFN-γ and GM-CSF in the TCM did not necessarily correlate with the frequency of hybrid neutrophils (>10% of all TANs) in each tumor as shown in Figures 3E and 3F. Also, when we exposed BMNs from the same donor to different hybrid-inducing TCMs containing variable concentrations of IFN-γ and GM-CSF, we were also unable to observe a clear relationship between absolute levels of GM-CSF and IFN-γ and the degree of hybrid neutrophil formation (Figure S3B). These data suggest that there is a requisite threshold level of GM-CSF and IFN-γ, and additional tumor-derived factors may contribute to the process of hybrid neutrophil differentiation.

CD11b$^+$CD15^{hi}CD10$^-$CD16$^{int/low}$ Progenitors Give Rise to APC-like Hybrid Neutrophils

The low frequency of APC-like hybrid TANs along with high heterogeneity in their accumulation in cancer patients suggested that there might be precursor cells that could differentiate into this unique subset of neutrophils under specific favorable conditions in some tumors. Therefore, we sought to determine whether the ability of long-lived immature BMNs to develop hybrid neutrophils is either shared by all immature subsets or limited to a specific differentiation stage.

To address this question, we used the combined expression of the CD11b, CD15, CD10, CD49d, and CD16 to distinguish the different maturational states of BMNs (Elghetany, 2002). As expected, we found that CD11b$^+$CD15^{hi} BMNs consist of a heterogeneous combination of mature CD16^{hi}CD10$^+$CD49d$^-$ cells, immature CD16^{int}CD10$^-$CD49d$^-$ band cells, and CD16$^{low/-}$CD10$^-$CD49d$^+$ metamyelocytes/myelocytes (Figure 4A; expression of CD49d is not shown). Of note, all mature and immature BMNs express CD66b but at slightly different levels (Figure S4A). The detailed phenotype of neutrophils at different maturation stages is summarized in Figure S4B. We isolated BMNs at different stages of maturation by flow cytometry sorting based on these phenotypes. Cytomorphology confirmed that each population was associated with distinct maturation stages (Figure 4B). These sorted subsets of BMNs were cultured in the presence of low concentration of IFN-γ (50 pg/ml) and GM-CSF (50 pg/ml) for 6 days, after which the resulting CD11b$^+$CD15^{hi}CD66b$^+$ neutrophil populations were analyzed for surface expression of CD14 and HLA-DR (Figure 4C). Our data revealed that CD14$^+$HLA-DR$^+$ hybrid neutrophils could be generated from all immature stages of neutrophils except the terminally differentiated, mature, segmented neutrophils. However, the

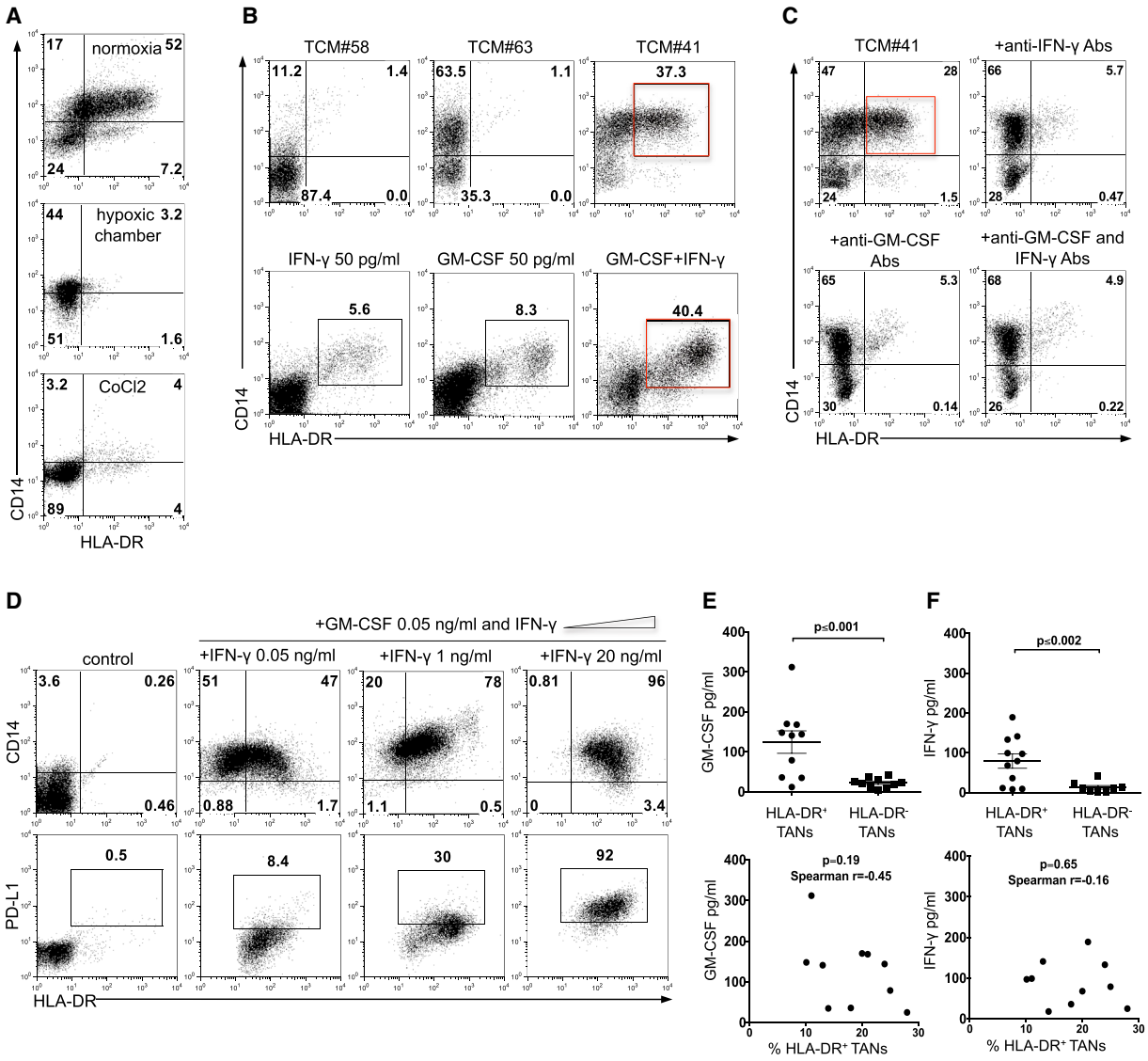

Figure 3. Tumor-Derived IFN-γ and GM-SCF Synergistically Differentiate Immature Neutrophils into a Subset of APC-like Hybrid Neutrophils

(A) Flow cytometric analysis of CD14 and HLA-DR expression on gated live CD11b$^+$CD15^{hi}CD66b$^+$ BMNs cultured in the presence of hybrid-inducing TCM under normoxic and hypoxic cell culture conditions.

(B) Flow cytometric analysis of CD14 and HLA-DR expression on gated live CD11b$^+$CD15^{hi}CD66b$^+$ BMNs cultured in the presence of different TCMs (upper panel) or with IFN-γ and/or GM-CSF (lower panel).

(C) The effect of IFN-γ and GM-CSF blocking Abs (5 μg/ml) in blunting the formation of HLA-DR$^+$CD14$^+$ hybrid neutrophils in vitro (red box).

(D) The expression of CD14 and HLA-DR markers on live CD11b$^+$CD15^{hi}CD66b$^+$ BMNs (upper panel) and PD-L1 on gated HLA-DR$^+$CD14$^+$ hybrid neutrophils (lower panel) differentiated with GM-CSF (50 pg/ml) and increasing doses of IFN-γ in vitro.

(E and F) Levels of IFN-γ (E) and GM-CSF (F) in supernatants collected from the cell culture of small-sized tumor digests where APC-like hybrid TANs were or were not previously detected (set-off was >10% among all TANs) (line represents mean ± SEM, n = 10, Mann-Whitney test for unpaired data). Lower panels represent the correlation between the absolute levels of IFN-γ and GM-CSF in the TCM, with the frequency of hybrid neutrophils in each tumor shown in the upper graphs. Non-parametric Spearman test was used to determine the degree of correlation.

Representative dot plots from one of five experiments are shown in (A–D). See also Figure S3.

level of HLA-DR expression on these hybrid neutrophils was affected by the degree of immaturity of the neutrophils prior to exposure to IFN-γ and GM-CSF: the more mature CD15^{hi}CD10$^-$CD16^{int} band cells gave rise to hybrid neutrophils, with the highest expression of HLA-DR on the surface when compared with hybrid neutrophils differentiated from CD15^{hi}CD10$^-$CD16$^{-/lo}$ myelocytes and metamyelocytes/early bands (Figure 4C). Interestingly, the majority of the neutrophils differentiated from CD15^{hi}CD10$^-$CD16^{int} band cells were able to change their nuclear contour from band-like to oval when compared with neutrophils differentiated from CD15^{hi}CD10$^-$CD16$^{-/lo}$ myelocytes and metamyelocytes/early bands (Figure 4D).

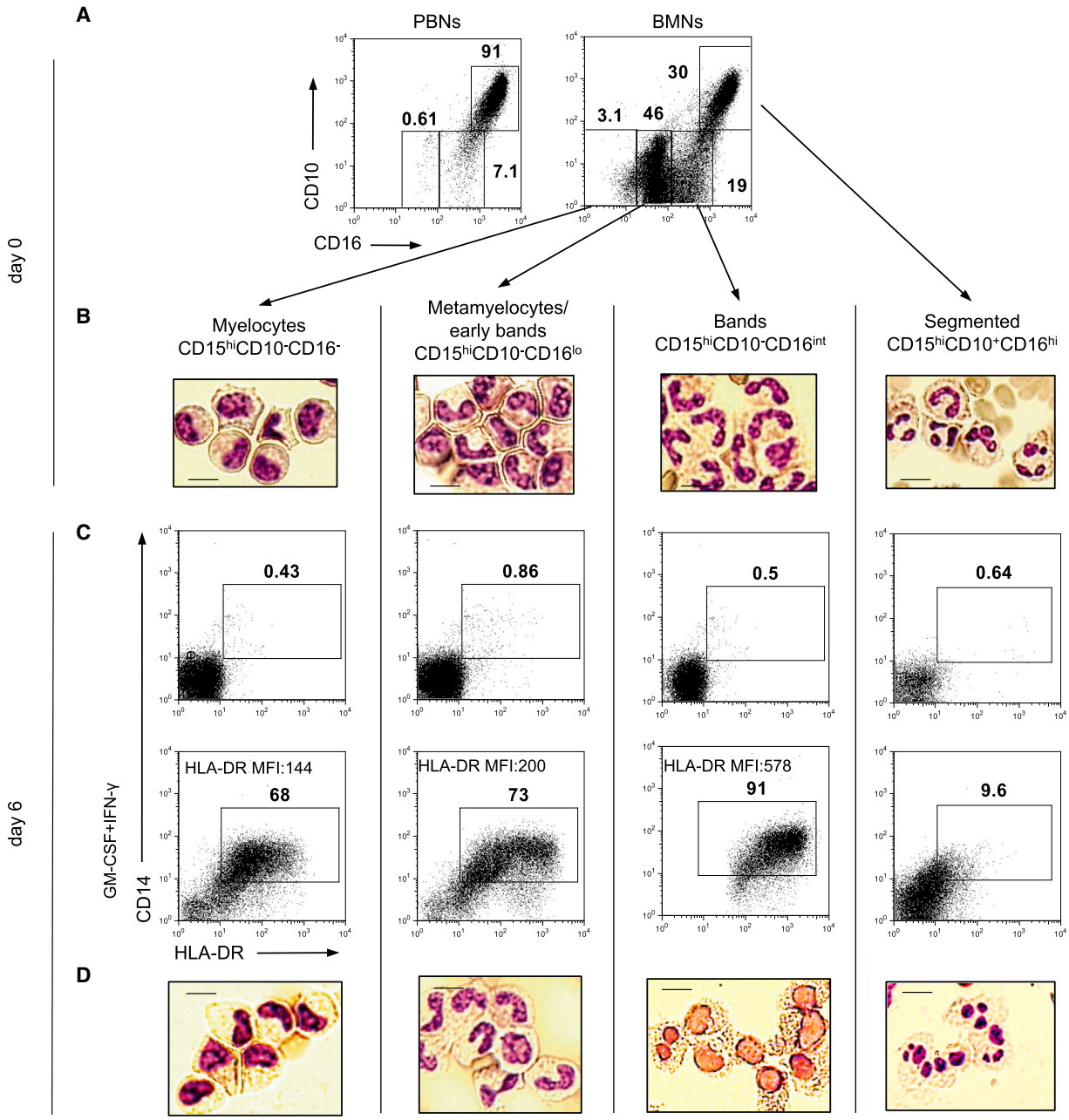

Figure 4. APC-like Hybrid Neutrophils Originate from CD11b$^+$CD15^{hi}CD66b$^+$CD10$^-$CD16$^{lo/int}$ Progenitors

(A) Flow cytometric analysis of the expression of CD10 and CD16 on gated live CD11b$^+$CD15^{hi}CD66b$^+$ neutrophils isolated from peripheral blood (PBNs) and bone marrow (BMNs) of cancer patients.

(B) Cytospins were made from sorted BMNs at different stages of maturation and stained with the Hema3 Stat Pack Kit (Wright-Giemsa-like stain).

(C) Sorted BMNs at different stages of maturation were differentiated in the presence of IFN-γ (50 pg/ml) and GM-CSF (50 pg/ml) in vitro. Expression of HLA-DR and CD14 markers was analyzed by flow cytometry on CD11b$^+$CD15^{hi}CD66b$^+$ BMNs.

(D) Cytomorphology of APC-like HLA-DR$^+$ hybrid neutrophils differentiated from the sorted populations of BMNs at different stages of maturation.

Representative results from one of four experiments are shown in (A–D). Scale bar, 10 μm. See also Figure S4.

Importantly, the circulating blood CD16$^{int/lo}$CD10$^-$ immature neutrophils that could potentially traffic into tumors were also able to differentiate into hybrid neutrophils in the presence of hybrid-inducing TCM or IFN-γ and GM-CSF (Figure S4C).

Ikaros Negatively Regulates the Development of APC-like Hybrid Neutrophils

Murine models have shown that the transcription factor Ikaros is involved in the control of neutrophil differentiation by silencing specific pathways in common precursors that allow

for macrophage-monocyte development (Dumortier et al., 2003; Papathanasiou et al., 2003). Given that hybrid neutrophils exhibit some characteristics of monocytic lineage cells, but can be differentiated from granulocyte-committed precursors, we hypothesized that the hybrid-inducing ability of TCM may be due to two possible synergistic effects on granulocyte progenitor cells: (1) premature downregulation of Ikaros, thus allowing some degree of monocyte differentiation to occur; and (2) the provision of the appropriate macrophage stimulating factors (i.e., GM-CSF) to activate the monocyte differentiation pathways.

We measured the level of Ikaros expression in BMNs at different stages of maturation and found that Ikaros was upregulated in all immature neutrophils (bands and metamyelocytes), with lower levels in mature BMNs and PBNs (Figure 5A). The analysis of BMNs treated with hybrid-inducing TCM revealed that the Ikaros level was lower in HLA-DR$^+$ hybrid BMNs compared with HLA-DR$^-$ canonical BMNs (Figure 5B). Thus hybrid-inducing TCM induced premature downregulation of Ikaros in HLA-DR$^+$ hybrid BMNs. We next cultured BMNs with hybrid-inducing TCM in the presence or absence of the drug lenalidomide, which causes proteasomal degradation of the human Ikaros proteins (Kronke et al., 2014). The addition of lenalidomide to TCM-treated BM neutrophils dramatically facilitated the development of HLA-DR$^+$CD14$^+$ hybrid neutrophils (Figure 5C). Together, these data suggest that Ikaros negatively regulates this process in the presence of tumor-derived factors.

We then measured the level of Ikaros in BMN progenitors incubated with or without low-dose IFN-γ and/or GM-CSF at days 1, 3, and 5. Downregulation of Ikaros was only observed when both IFN-γ and GM-CSF were present for at least 5 days, confirming their synergistic effect in this process (Figure 5D). Next, we downregulated Ikaros in BMNs by adding lenalidomide and cultured these cells with either IFN-γ or GM-CSF. The incubation of BMNs with the combination of GM-CSF and lenalidomide, but not IFN-γ and lenalidomide, resulted in efficient development of HLA-DR$^+$CD14$^+$ hybrid cells (80%–90% among all BMNs) (Figure 5D). These data confirm the hypothesis that the premature downregulation of Ikaros in concert with the macrophage stimulatory factor GM-CSF are requisite for the development of hybrid neutrophils from neutrophil progenitors.

BM-Derived Hybrid Neutrophils Recapitulate the Function of APC-like Hybrid TANs

Next, we investigated whether the BM-derived hybrid neutrophils also functionally resemble hybrid TANs in their ability to stimulate T cell responses. For this purpose, we differentiated immature BMNs into activated canonical and hybrid neutrophils (Figure S5A) and co-cultured them with autologous PBMCs stimulated with plate-bound anti-CD3 Abs. We found that both subsets of neutrophils augmented the expression of activation markers CD25 and CD69 on stimulated T cells to the same degree (Figure S5B). However, HLA-DR$^+$ hybrid neutrophils exerted a significantly stronger stimulatory effect on T cell proliferation and IFN-γ production than the canonical neutrophils (Figures 6A and 6B). The BM-derived hybrid neutrophils differentiated with low doses of IFN-γ and GM-CSF also recapitulated the T cell stimulatory activity of hybrid TANs (Figure 6A). However, as described above, the treatment of BMNs with a low dose of GM-CSF and IFN-γ at concentrations more than 1 ng/ml gradually induced the expression of PD-L1 on the HLA-DR$^+$ BMNs (Figure 3D, lower panel). When we co-cultured these PD-L1$^+$HLA-DR$^+$ BMNs with autologous PBMCs stimulated with anti-CD3 Abs, we found marked suppression of T cell proliferation (Figure 6C, upper panel), which was substantially inhibited by PD-L1 blocking Abs (Figure 6C, lower panel). Thus, high doses of IFN-γ can convert the T cell stimulatory HLA-DR$^+$ BMNs into a suppressive population via upregulation of PD-L1. These results demonstrate some functional plasticity in the APC-like neutrophils.

To determine whether the hybrid neutrophils are able to induce the proliferation of allogeneic T cells in a mixed-lymphocyte reaction, we co-cultured BM-derived hybrid and canonical neutrophils with allogeneic T cells purified from the peripheral blood of healthy donors. BrdU incorporation assays revealed that hybrid neutrophils, but not canonical neutrophils, were able to initiate the allogeneic proliferation of both CD4 and CD8 cells (Figure 6D). In addition, similar to hybrid TANs, BM-derived hybrid neutrophils pulsed with a peptide pool of viral antigens were able to initiate the autologous memory CD8 and CD4 cell response more efficiently than canonical neutrophils (Figure S5C). These data demonstrate the functional resemblance between BM-derived and tumor-derived hybrid neutrophils, and justify the use of this model to investigate additional functions of this rare subset of TANs.

APC-like Hybrid Neutrophils Stimulate and Augment Anti-Tumor Effector T Cell Responses

Next, we evaluated the effect of canonical and hybrid neutrophils on anti-tumor effector T cells using a newly developed in vitro model. We transduced human T cells with a high-affinity transgenic T cell receptor (TCR) called Ly95 that recognizes an HLA-A*0201-restricted peptide sequence in the human cancer testis antigen, NY-ESO-1 (Moon et al., 2016). As target cells, we used a genetically modified A549 human lung adenocarcinoma cell line expressing the NY-ESO-1 protein in the context of HLA-A*0201 (A549 A2-NY-ESO-1 cells) (Moon et al., 2016). Co-culturing of Ly95 T cells with A549 A2-NY-ESO-1 tumor cells resulted in robust production of IFN-γ and Granzyme B in Ly95 T cells (Figure 7A). When we added BM-derived hybrid neutrophils into this system, the production of IFN-γ and Granzyme B in Ly95 T cells was markedly elevated (Figures 7A and 7B) and increased compared with canonical neutrophils. Of note, the addition of the hybrid neutrophils into Ly95 T cells co-cultured with control A549 cells did not induce the production of these factors, indicating that hybrid neutrophil-mediated stimulation of Ly95 cells was NY-ESO-1 specific and not the result of allostimulation (data not shown).

Using a transwell assay system, we found that HLA-DR$^+$ hybrid BMNs induced the stimulation of IFN-γ production by Ly95 T cells only when the cells were in direct contact (Figure S5D). Since hybrid BMNs are characterized by increased expression of co-stimulatory molecules OX40L, 4-1BBL CD86, and CD54 (Figures 2D, 2E, and S2D), we co-cultured Ly95 T cells with A549 A2-NY-ESO-1 tumor cells and with hybrid BMNs in the presence of blocking Abs to these upregulated co-stimulatory molecules. Figure 7A shows a representative experiment in which the stimulatory effect of hybrid neutrophils

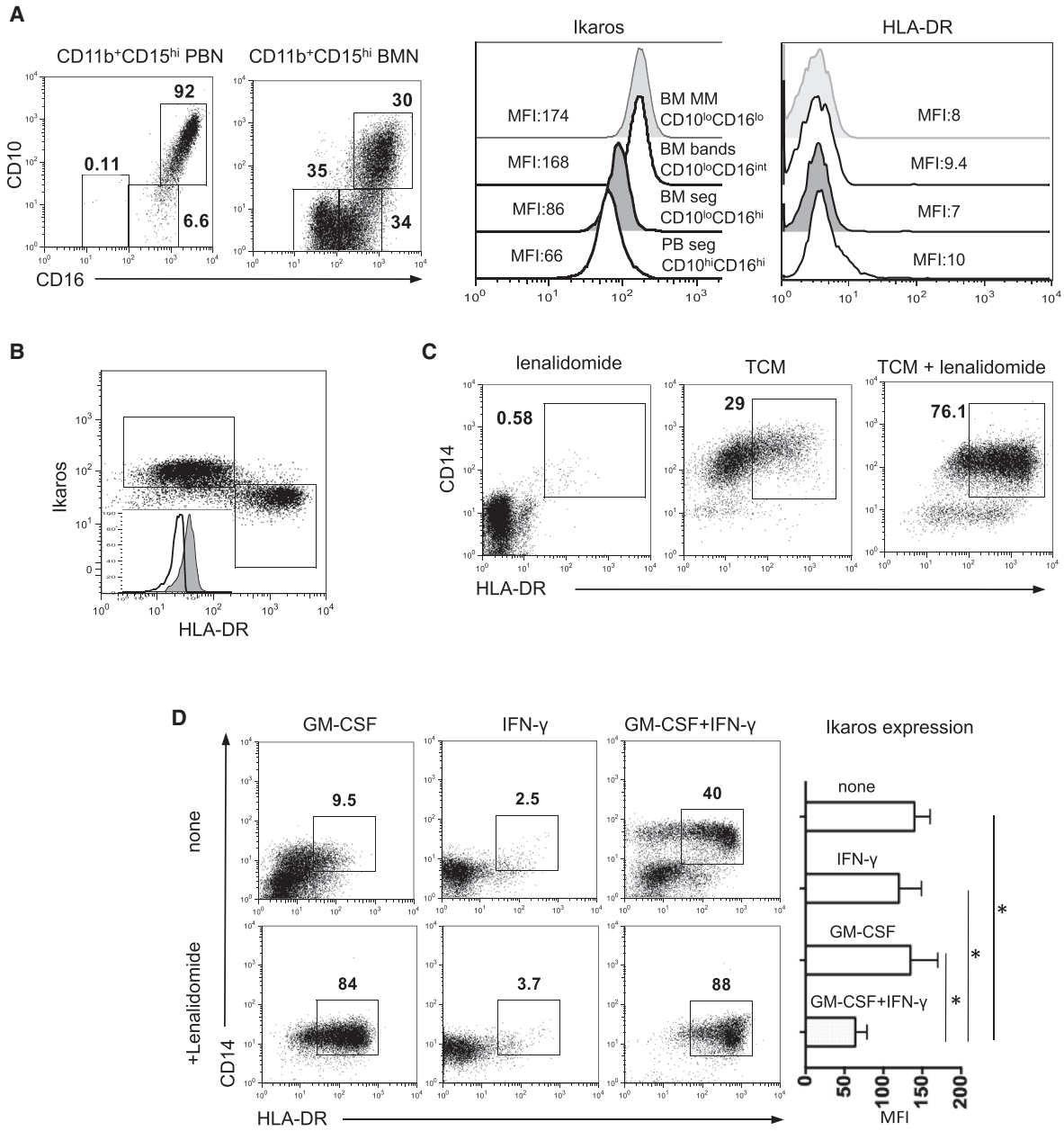

Figure 5. Transcription Factor Ikaros Negatively Regulates the Differentiation of Hybrid Neutrophils

(A) Flow cytometric analysis of the level of Ikaros and HLA-DR expression in PBNs and BMNs at different stages of maturation. Results are shown as mean fluorescence intensity (MFI).

(B) Flow cytometric analysis of the level of Ikaros expression in the HLA-DR$^+$ hybrid and HLA-DR$^-$ canonical CD11b$^+$CD15^{hi}CD66b$^+$ BMNs.

(C) Flow cytometric analysis of CD14 and HLA-DR expression on gated live CD11b$^+$CD15^{hi}CD66b$^+$ BMNs cultured in the presence of lenalidomide (10 μM) and hybrid-inducing TCM (30% v/v) for 6 days.

(D) The effect of IFN-γ (50 pg/ml) and GM-CSF (50 pg/ml) on the formation of HLA-DR$^+$CD14$^+$ hybrid neutrophils in the absence (upper panel) or presence (lower panel) of lenalidomide (10 μM) in vitro. The level of Ikaros expression (MFI) in BMNs treated with IFN-γ (50 pg/ml) and GM-CSF (50 pg/ml) for 5 days is shown (mean ± SEM, n = 3, *p ≤ 0.01, Wilcoxon matched-pairs rank test).

Representative dot plots from one of six experiments are shown in (A–D).

was partially abrogated in the presence of anti-CD54, 4-1BBL, OX-40L, and CD86 blocking Abs (Figure 7A).

Next we asked whether APC-like hybrid neutrophils could directly trigger NY-ESO-1 specific response of Ly95 cells. Given that Ly95 cells specifically recognize the HLA-A*02-restricted peptide of NY-ESO-1, we pulsed HLA-A*02$^+$ BM-derived canonical and hybrid neutrophils with the NY-ESO-1 (157–165, SLLMWITQV) peptide and then co-cultured the exposed neutrophils with Ly95 T cells for 24 hr. We found that hybrid HLA-A*02$^+$HLA-DR$^+$ hybrid neutrophils preloaded with the peptide

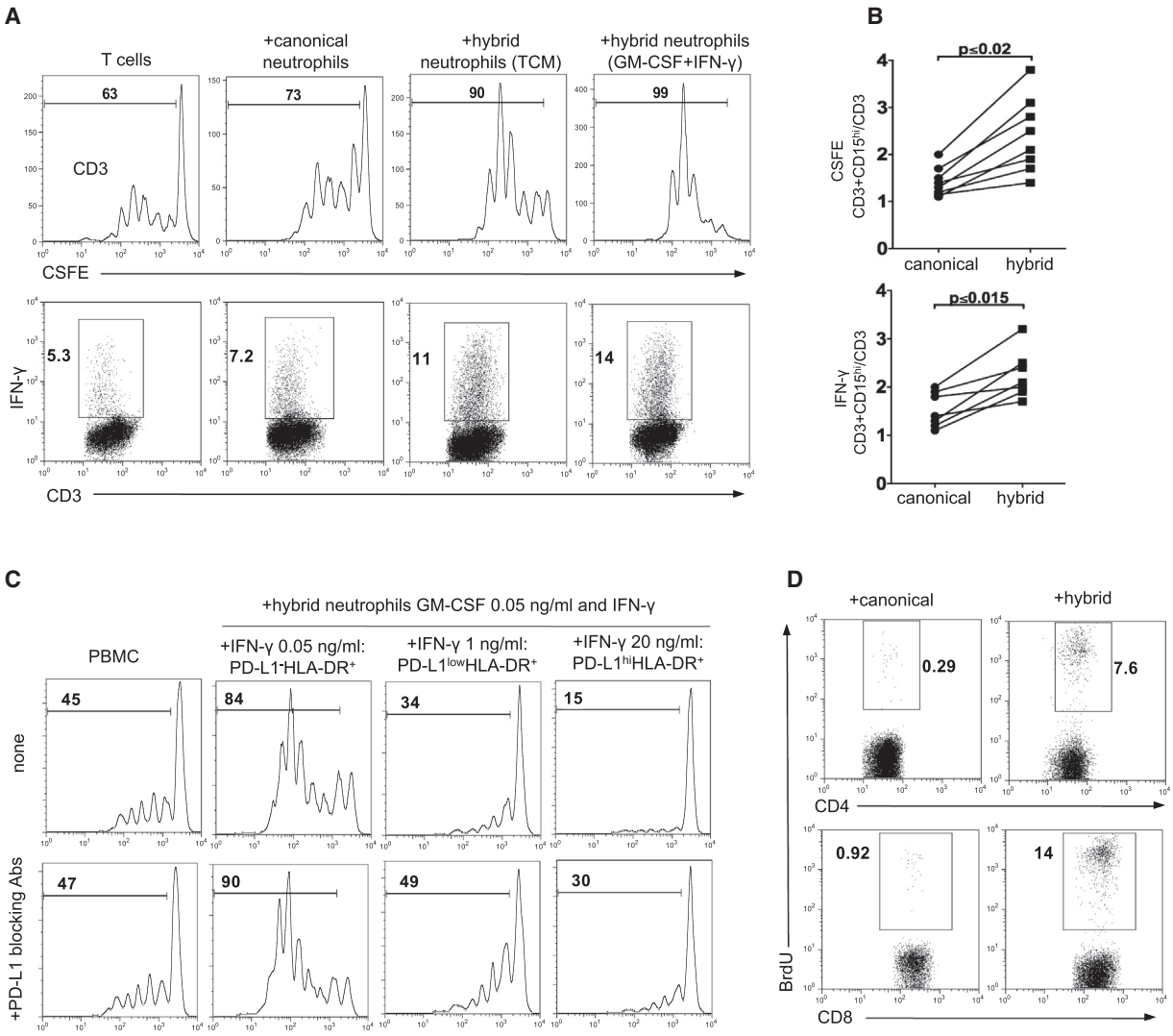

Figure 6. APC-like Hybrid Neutrophils Stimulate Antigen-Nonspecific T Cell Responses

(A) The proliferation and IFN-γ production of anti-CD3 Abs stimulated autologous T cells in the presence of BM-derived canonical and hybrid neutrophils differentiated with hybrid-inducing TCM or IFN-γ (50 pg/ml) and GM-CSF (50 pg/ml).

(B) Summary results of autologous T cell proliferation (upper graph) and IFN-γ production (lower graph) in the presence of canonical and hybrid neutrophils. Data are presented as a ratio (CD3 cells + $CD15^{hi}$)/(CD3) (n = 8, Wilcoxon matched-pairs rank test).

(C) The proliferation of CFSE-labeled autologous PBMCs cultured with hybrid BMNs with different level of PD-L1 expression in the presence (lower panel) or absence PD-L1 blocking Abs (5 μg/ml) (upper panel). PD-$L1^{-/lo/hi}$HLA-DR^{+} hybrid neutrophils were differentiated with GM-CSF (50 pg/ml) and increasing doses of IFN-γ.

(D) The proliferation of allogeneic T cells from healthy donors in the presence of APC-like hybrid neutrophils in a mixed-lymphocyte reaction.

Representative results from one of six experiments are shown in (C) and (D). See also Figure S5.

triggered IFN-γ production in Ly95 T cells more effectively than peptide-loaded canonical neutrophils (Figure 7C). These data demonstrate that hybrid neutrophils can trigger and significantly augment the activation of antigen-specific effector T cells.

APC-like Hybrid Neutrophils Are Able to Cross-Present Tumor Antigens

The assays described above evaluated the ability of the TANs to present antigenic peptides, but did not address the ability of hybrid neutrophils to process antigens, as the high-affinity major histocompatibility (MHC) class I binding peptides could bind directly to the surface MHC class I and do not require uptake and processing. We therefore performed experiments with DQ ovalbumin (DQ-OVA) and demonstrated that hybrid neutrophils were able to take up and process ovalbumin to a higher degree than canonical neutrophils (Figure 7D). To evaluate whether hybrid neutrophils are able to present extracellular protein to effector CD8 cells (cross-presentation), we preloaded HLA-A*02-positive BM-derived hybrid and canonical neutrophils with full-length NY-ESO-1 protein and mixed them with Ly95 cells for 24 hr (Figure 7E). We found that these canonical and hybrid neutrophils were not sufficient to trigger Ly95 T cell response.

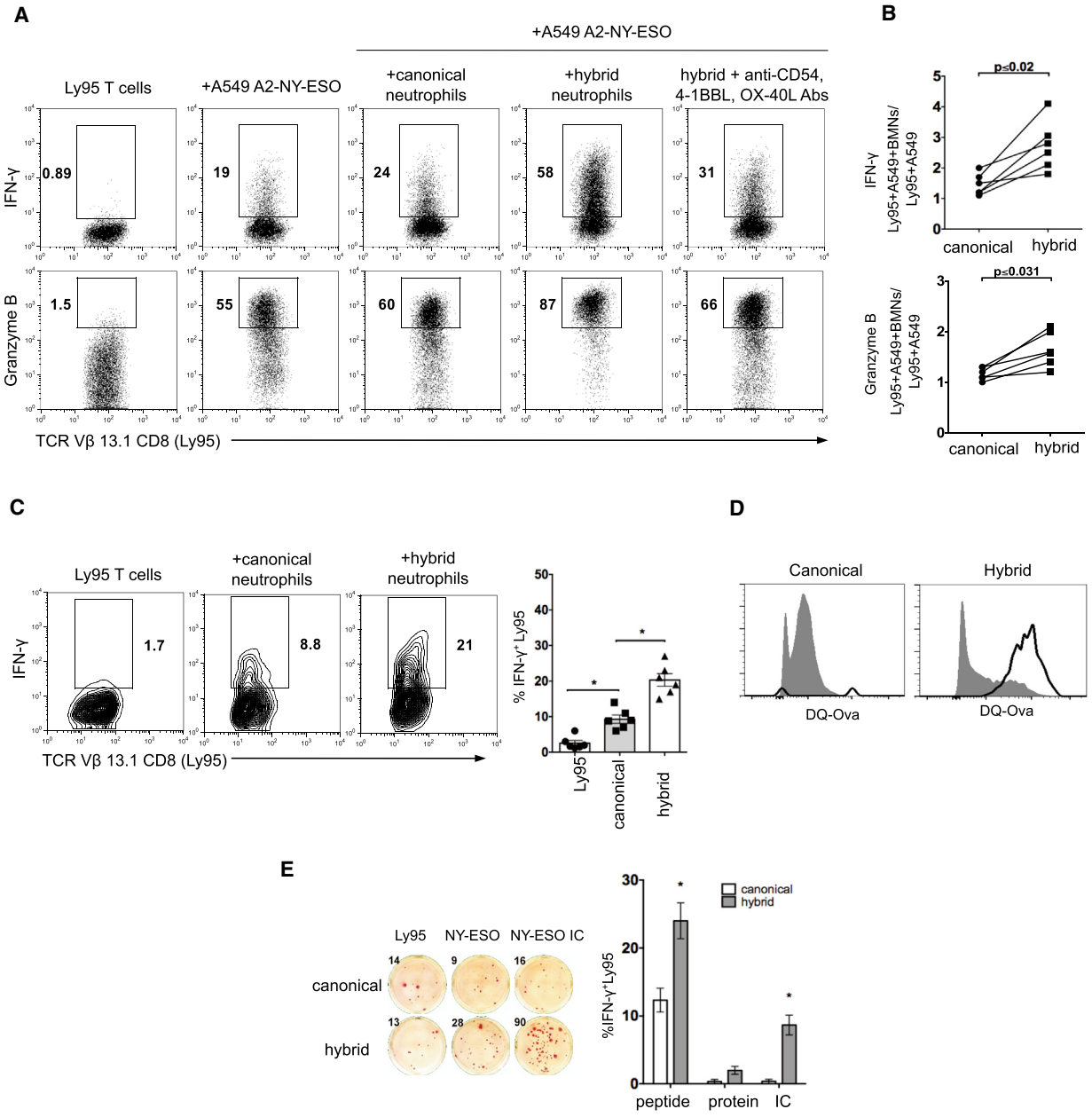

Figure 7. APC-like Hybrid Neutrophils Are Able to Trigger and Stimulate NY-ESO-Specific Effector T Cell Responses

(A) NY-ESO-specific Ly95 cells (TCR Vβ13.1$^+$CD8$^+$) were stimulated with A549 tumor cell line expressing NY-ESO-1 in the context of HLA-A*02 (A2/NY-ESO-1 A549) in the presence of BM-derived canonical and hybrid neutrophils. Intracellular IFN-γ and Granzyme B production was measured by flow cytometry.

(B) Cumulative results showing the Ly95 cell stimulatory activity of canonical and hybrid neutrophils. Stimulatory activity was defined as a ratio (Ly95 cells + A549-NY-ESO + BMN)/(Ly95 cells + A549-NY-ESO) (n = 6, Wilcoxon matched-pairs rank test).

(C) HLA-A02$^+$ canonical or hybrid neutrophils were pulsed with synthetic NY-ESO-1 peptide and co-cultured with Ly95 cells for 24 hr. Intracellular IFN-γ was assessed by flow cytometry (mean ± SEM, n = 6, *p ≤ 0.01, Wilcoxon matched-pairs rank test).

(D) DQ-OVA uptake and processing by BM-derived canonical or hybrid neutrophils (open histograms). Cells incubated at 4°C served as controls (shaded histograms).

(E) Cross-presentation of NY-ESO-1 epitopes to Ly95 cells by HLA-A02$^+$ canonical or hybrid neutrophils preloaded with NY-ESO-1 protein, NY-ESO-1 peptide, or NY-ESO-immune complex (IC). IFN-γ ELISpot (mean ± SEM, n = 6, *p ≤ 0.01 canonical versus hybrid, Wilcoxon matched-pairs rank test).

Ly95 T cells mixed with control, unloaded neutrophils generated a low background of IFN-γ-positive spots due to endogenous activity of Ly95 cells from the prior CD3 stimulation required for expansion of these cells after TCR transduction (Figure 7E).

Next, we sought to employ the Fc receptors (FcγR) that are highly expressed on hybrid neutrophils (Figure 2D) and deliver the NY-ESO-1 protein as an immunoglobulin G (IgG)-immune complex to trigger the more efficient FcγR-mediated antigen

uptake and presentation. For this purpose, we pre-exposed the neutrophil subsets to NY-ESO-1 immune complexes formed by incubating the NY-ESO-1 protein with anti-NY-ESO-1 monoclonal Abs and mixed them with Ly95 cells for 24 hr. Under these conditions, we observed that hybrid neutrophils, but not canonical neutrophils, were able to cross-present NY-ESO epitopes and induce low-level, but NY-ESO-specific, production of IFN-γ by Ly95 T cells (Figure 7E). These data demonstrate that hybrid neutrophils have the ability to take up and cross-present exogenous tumor antigens, at least under the conditions used here.

DISCUSSION

We identified a subset of TANs that exhibited the hybrid phenotypic and functional characteristics of neutrophils and APCs. These APC-like hybrid TANs were superior to canonical TANs in their ability to induce and stimulate anti-tumor T cell responses. We identified the progenitors of APC-like hybrid TANs, along with the tumor-derived and transcriptional factors responsible for the development of these cells. Thus, our findings demonstrate that the early-stage lung tumor microenvironment can drive neutrophils to differentiate into a cell subset with enhanced anti-tumor capabilities.

It has been previously recognized that neutrophils can acquire characteristics of "professional APCs" under certain non-cancerous physiological and pathological conditions (Ashtekar and Saha, 2003). Specifically, neutrophils have been reported to upregulate MHC class II molecules and co-stimulatory molecules, such as CD80 and CD86, in response to inflammatory cytokines and during autoimmune pathology or inflammatory diseases (Iking-Konert et al., 2005; Wagner et al., 2006). There have also been several reports demonstrating the ability of neutrophils to present viral and bacterial antigens to T cells, provide accessory signals for T cell activation, and prime antigen-specific Th1 and Th17 cells (Abi Abdallah et al., 2011; Radsak et al., 2000; Tvinnereim et al., 2004). Little is known, however, about factors that trigger the formation and origin of these cells, and the precise functional capacities they possess, especially in humans. Moreover, the detailed phenotype and function of these APC-like neutrophils have not yet been reported and studied in cancer patients. The cells most similar to hybrid TANs appear to be those recently identified by Geng et al. (2013) and Matsushima et al. (2013), who described the ability of a subset of murine neutrophils to acquire markers of dendritic cells (CD11c, MHC II, CD80, and CD86) and termed these cells "neutrophil-DC hybrids." However, the phenotype of these neutrophil-DC hybrids is somewhat different from APC-like hybrid TANs that exhibit a partial phenotype of DC (MHC class II, CD86, CCR7) along with a partial phenotype of monocytes/macrophages (CD14, CD206, $CD64^{hi}$, $CD32^{hi}$), and lacked other defining markers of dendritic cells and macrophages such as CD209, CD204, CD83, CD80, CD1c, CD163, and CCR6.

Over the last decade there has been an increasing focus on the interactions between inflammatory myeloid cells and T cells within the tumor microenvironment. In this study, we identified a specialized neutrophil subpopulation enabling augmentation of both antigen non-specific and tumor-specific T cell responses by providing co-stimulatory signals through the OX40L, 4-1BBL CD86, and CD54 molecules. Our data are concordant with previous studies showing that activated granulocytes can provide accessory signals for T cell activation (Radsak et al., 2000). We also found evidence for functional plasticity in the generation of APC-like neutrophils in terms of regulation of T cell response. Differentiation of immature neutrophils with low doses of IFN-γ resulted in highly immunostimulatory cells; however, high doses of IFN-γ resulted in formation of $PD\text{-}L1^{hi}$ hybrid neutrophils that profoundly suppressed T cell responses. This dichotomous in vitro effect of neutrophils on T cell response may suggest an important role of hybrid neutrophils in the regulation of the normal physiological inflammatory processes, whereby T cell stimulation needs to be followed by suppression to resolve an inflammatory process.

One of the key functional findings of our study was that APC-like hybrid TANs acquired new functions compared with canonical TANs and were able to take up, degrade, and cross-present tumor antigens. Cross-presentation was triggered in hybrid neutrophils when NY-ESO-1 protein was delivered as an IgG-immune complex; however, this cross-presentation occurred at a relatively low level. These data suggest that the hybrid neutrophils can take up and process antigens by means of the high-affinity IgG receptors FcγRI and FcγRII, which are highly expressed on hybrid neutrophils compared with canonical neutrophils. These hybrid neutrophils may "regurgitate" processed peptide outside of the cell (Potter and Harding, 2001) and thus facilitate the antigen uptake and processing by other professional APCs. Our data are consistent with previous studies showing the critical role of FcγR in enhancing the cross-presentation of NY-ESO-1 by professional APCs (Nagata et al., 2002).

The ability of human neutrophils to express some APC markers in vitro after stimulation with inflammatory factors such as macrophage CSF, IFN-γ, GM-CSF, TNF-α, IL-3, IL-1β, and IL-4 was discovered over a decade ago (Gosselin et al., 1993; Reinisch et al., 1996; Oehler et al., 1998). For example, the combination of GM-CSF, IL-4, and TNF-α induced the expression of MHC II, CD40, CD86, CD1a, CD1b, and CD1c, whereas the combination of GM-CSF, TNF-α, and IFN-γ triggered MHC II, CD80, CD83, and CD86 expression by human neutrophils (Iking-Konert et al., 2001; Oehler et al., 1998). Moreover, it has been reported that neutrophils could even be reprogrammed and transdifferentiated into macrophages and DC after co-culture with multiple cytokines in vitro (Araki et al., 2004; Iking-Konert et al., 2005; Koffel et al., 2014). It should be noted, however, that high (generally non-physiologic) concentrations of inflammatory factors (ranging from 1 to 100 ng/ml) were used in all these previous studies to induce the upregulation of APC markers, and differentiation could even be observed in mature end-stage neutrophils. It seems unlikely that neutrophils would encounter these artificial conditions in vivo and it has been unknown whether these factors are required and sufficient for the generation of APC-like neutrophils in vivo. We found that the tumors containing an increased number of hybrid TANs secreted many of the inflammatory factors described above at very low concentrations when compared with tumors without hybrid TANs. When tested, however, only IFN-γ and GM-CSF at the low

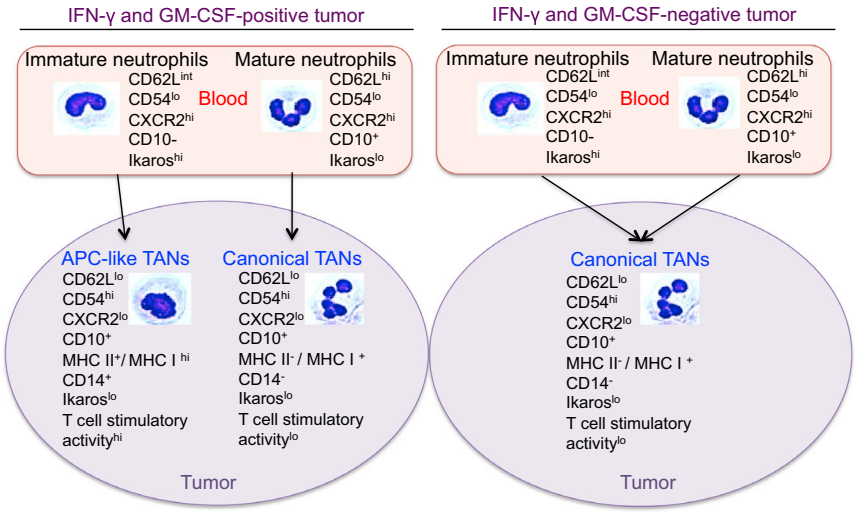

Figure 8. Schematic Model of Neutrophil Differentiation in Early-Stage Human Lung Cancer

concentrations found in TCM were able to synergistically induce hybrid neutrophils. The development of APC-like neutrophils occurred over a 5-day time period and required the synthesis of APC receptors de novo in the long-lived $CD10^{-}CD16^{low/int}$ immature neutrophils, suggesting that hybrid neutrophils are a result of a differentiation process and not simply an acute activation event.

Mechanistically, we also found that IFN-γ and GM-CSF exert their APC-promoting effects on immature neutrophils partially via the downregulation of the transcription factor Ikaros. Although Ikaros is well known as a key regulator of lymphocyte differentiation (O'Brien et al., 2014), our data suggest that Ikaros has a much broader role in hematolymphoid differentiation. Our results are consistent with previous studies in murine cells demonstrating that Ikaros controls neutrophil differentiation by silencing of genes that are necessary for macrophage-monocyte development (Papathanasiou et al., 2003).

Although the in vitro conditions used in our experiments may not necessarily reflect what actually transpires in vivo, we propose a model of human neutrophil differentiation in different lung tumor microenvironments at early stages (Figure 8). According to this model, neutrophils adapt differently to the tumor microenvironment. Mature neutrophils recruited into tumors acquire the phenotype of highly activated neutrophils, resulting in the formation of canonical TANs. However, the immature neutrophils, which circulate to varying degrees in cancer patients, are more plastic. When immature neutrophils are recruited into tumors which produce appropriate levels of IFN-γ and GM-CSF, they change their differentiation program and give rise to a subset of APC-like hybrid TANs exhibiting composite characteristics of neutrophils and APCs. However, if the level of IFN-γ and GM-CSF is not significant enough to initiate the differentiation of immature neutrophils into the hybrid neutrophils, or other inhibitory factors or conditions are present in the tumor (i.e., hypoxia), the immature neutrophils use a default pathway of differentiation and become activated canonical TANs.

The concept of neutrophil diversity and plasticity has begun to emerge in a variety of inflammatory disorders and murine tumor models; however, to date there has been no convincing evidence showing that specialized neutrophil subpopulations with different functions exist in human cancers. Our data suggest that the tumor microenvironment in some small, early-stage lung tumors can induce the formation of an immunostimulatory subset of neutrophils that have the ability to activate T cells and, in a general way, resemble the N1 neutrophil phenotype previously described in mice (Fridlender et al., 2009). By gaining a better understanding of the cellular and molecular processes in early-stage cancers that control tumor growth, it may be possible to enhance or mimic these factors and conditions to potentially boost natural or vaccine-induced anti-tumor immunity.

EXPERIMENTAL PROCEDURES

Study Design

A total of 109 patients with stage I–II lung cancer, who were scheduled for surgical resection, consented to tissue collection of a portion of their tumor and/or blood for research purposes at the Hospital of the University of Pennsylvania and The Philadelphia Veterans Affairs Medical Center, which had been approved by their respective Institutional Review Boards. Detailed characteristics of the patients can be found in the Supplemental Experimental Procedures and Table S1.

Preparation of a Single-Cell Suspension from Lung Tumor Tissue

We used our optimized disaggregation method for fresh human lung tumors that preserves the phenotype and function of the immune cells as previously described in detail (Quatromoni et al., 2015).

Neutrophil Isolation

TANs were isolated from tumor single-cell suspensions using positive selection of $CD15^{+}$ or $CD66b^{+}$ cells with microbeads as previously described (Eruslanov et al., 2014). TAN subsets were flow sorted based on the phenotype of canonical ($CD11b^{+}CD66b^{+}CD15^{hi}HLA\text{-}DR^{-}$) and hybrid ($CD11b^{+}CD66b^{+}CD15^{hi}HLA\text{-}DR^{+}$) TANs. PBNs and BMNs were isolated from EDTA anticoagulated peripheral blood and BM single-cell suspension, respectively, using positive selection of $CD15^{+}$ or $CD66b^{+}$ cells with microbeads. See Supplemental Experimental Procedures for details.

Generation of BM-Derived Macrophages, Dendritic Cells, and Hybrid and Canonical Neutrophils

To differentiate BMNs into the cells that resemble canonical and hybrid TANs, we cultured the purified BMNs with a different type of TCM for 7 days. Alternatively, hybrid neutrophils were differentiated from BMNs with low doses of IFN-γ and GM-CSF. BM-derived macrophages and dendritic cells were generated by culturing $CD15^{-}CD11b^{+}$ BM cells with M-CSF or IL-4 and GM-CSF, respectively.

Flow Cytometry

Flow cytometric analysis was performed according to standard protocols. For phenotypic and functional analysis PBNs, BMNs, and TANs were gated on live $CD11b^{+}CD15^{hi}CD66b^{+}$ cells. For more details see Supplemental Experimental Procedures.

Antigen Non-specific T Cell Response

PBMCs or purified T cells were stimulated with plate-bound anti-human CD3 and/or anti-CD28 antibodies to induce antigen non-specific T cell response. For more details see Supplemental Experimental Procedures.

Virus-Specific Memory T Cell Response

Peripheral blood autologous T cells were used as responders and co-cultured with neutrophil subsets that had been pulsed with a mixture of viral peptides with a broad array of HLA types. The T cell response was quantified by IFN-γ ELISPOT.

NY-ESO-Specific T Cell Response

To study the regulation of antigen-specific effector T cell responses by neutrophil subsets, we used Ly95 TCR transduced T cells recognizing the HLA-A2 restricted NY-ESO-1:157–165 peptide antigen (Moon et al., 2016). Generation of NY-ESO-specific Ly95 TCR T cells and details of the procedures are provided in Supplemental Experimental Procedures.

Allogeneic Mixed-Lymphocyte Reaction

Purified allogeneic T cells from healthy donor PBMCs were used as responders and reacted with canonical or hybrid neutrophils (inducers) from lung cancer patients at a ratio of 1:1. Five days later, the proliferation of CD4 and CD8 T cells was measured using a BrdU incorporation assay.

Statistics

Comparisons between two groups were assessed with a two-tailed Student's t test for paired and unpaired data if data were normally distributed. Non-parametric Wilcoxon matched-pairs test and Mann-Whitney unpaired test were used when the populations were not normally distributed. Likewise, multiple groups were analyzed by one-way ANOVA with corresponding Tukey's multiple comparison test if normally distributed, or by the Kruskal-Wallis test with Dunn's multiple comparison test if not normally distributed. All statistical analyses were performed with GraphPad Prism 6. A p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2016.06.001.

AUTHOR CONTRIBUTIONS

S.S., S.A., and E.E. conceived the study. S.S., P.B., A.R., T.S., S.B., E.M., J.Q., C.D., M.F., and E.E. performed experiments and data analysis. Reagents were contributed by E.M. S.A., S.S., A.G., W.H., and J.C.-G. Manuscript preparation was performed by S.S. and E.E. Manuscript revisions were performed by S.A., W.H., and J.C.-G. All authors read and approved the final manuscript.

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Trends in Cancer

CellPress

Review

Mouse versus Human Neutrophils in Cancer: A Major Knowledge Gap

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Many types of cancer recruit neutrophils that could have protumor or antitumor effects on tumor development. Numerous findings in murine models suggest a predominantly protumoral role for neutrophils in cancer development. However, there are fundamental differences between mouse and human tumors in the evolution of tumors, genetic diversity, immune response, and also in the intrinsic biology of neutrophils that might have a profound impact on tumor development and the function of these cells. A crucial difference is that the majority of mouse tumor models lack the prolonged initial phases of multistage tumor evolution present in humans when antitumoral mechanisms are activated. In this review, we discuss the challenges specific to cross-species extrapolation of neutrophil function during mouse versus human tumor development.

Trends

Our current understanding of the role of neutrophils in tumor development has greatly depended on murine models of cancer.

Uncertainty exists regarding the phenotypes, functional roles, and relationships between different granulocytic cell populations during tumor progression.

The protumoral role of neutrophils in cancer development in mice is mostly associated with the idea of the development of PMN-MDSC.

The fundamental differences between mice and humans in the evolution of tumors, immune and inflammatory responses, genetic diversity, and intrinsic biology of neutrophils might have a profound impact on the function of neutrophils.

The detailed functions of tumor-associated neutrophils in human cancers remain to be determined.

Complexities in Understanding the Granulocytes in Human Cancer and Mouse Tumor Models

Neutrophils accumulate in many types of human and murine tumors and represent a significant portion of tumor-infiltrating myeloid cells [1–4]. There is growing evidence indicating that neutrophils have both protumor and antitumor effects on cancer development and this dual role has been comprehensively described in many recent reviews [5–9]. Importantly, however, our understanding of the role of neutrophils in tumor development has largely been based on observations made in murine models of cancer. Studies relying on these murine experimental systems have led investigators to postulate a protumoral and immunosuppressive role for neutrophils in cancer development, and this concept is the dominant view in the field. However, this perspective has been derived primarily from murine transplantable tumor models, which are associated with high tumor burden, minimal matrix, and rapid tumor growth. Importantly, these transplanted mouse tumor models use tumor cell lines that, by definition, grow well in mice and have therefore already undergone substantial cancer immunoediting and 'Darwinian' selection [10]. Thus, these models bypass the initial phases of tumor evolution that would be expected to occur during tumor development in humans. Given that, data obtained from these mouse models largely reflect the immune response as it occurs during the 'escape phase', where protumoral mechanisms already prevail. All these features are dissimilar to the initiation and slow evolution of human cancers, which occur over a much longer time period and therefore might have a profound impact on neutrophil phenotype and function in mouse tumors versus human cancers.

For these reasons, an unresolved issue in cancer biology is the nature and function of immune cells, including neutrophils, in human tumor tissues. In this review, we will point out the major

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obstacles and challenges that exist in the investigation of neutrophils in mouse tumor models and highlight the potential danger in extrapolating these findings to humans.

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In murine studies, it appears that tumor-associated neutrophils (TANs) exhibit both protumor and antitumor effects [5,11]. Multiple studies have shown that neutrophils promote tumor progression via matrix degradation, the secretion of protumor factors, direct stimulation of tumor cell proliferation, increased metastasis, and enhanced angiogenesis [7,12,13]. By contrast, antitumoral functions have also been reported [12,14]. Specifically, neutrophils have been reported to (i) induce tumor cell death via their potent antimicrobial killing machinery [15], (ii) enhance antibody-dependent cell-mediated cytotoxicity (ADCC) [16], (iii) produce factors to recruit and activate cells of the innate and adaptive immune systems [17], and (iv) boost T-cell responses following photodynamic and Bacillus Calmette–Guérin immunotherapy of cancer [18,19]. Given these varying effects of mouse TANs on tumor growth, the paradigm of antitumoral 'N1 neutrophils' versus protumoral 'N2 neutrophils' was proposed [11] and rapidly adopted.

Although the concept of neutrophil diversity and plasticity has begun to emerge in murine tumor models, uncertainty regarding the phenotypes, functional roles, and relationships between different granulocytic cell populations during tumor progression persists. For instance, N1 neutrophils have a hypersegmented nucleus characteristic of mature neutrophils, while N2 neutrophil populations consist of cells with a banded or ringlike nuclei, as seen in immature mouse neutrophils [11]. Given this fact, it remains possible that N1 and N2 neutrophils represent different stages of maturation rather than truly *bona fide* neutrophil phenotypic subtypes. Alternatively, it is very possible that, similar to our current understanding of the M1/M2 macrophage paradigm [20], TANs could represent a continuum of activation states, rather than two extremes.

Ongoing discussion continues regarding the relationship between neutrophils, TANs, and polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) during tumor progression, because PMN-MDSCs and neutrophils share a common set of markers and exhibit a degree of immunosuppressive capacity similar to N2 neutrophils [8,11,21,22]. Although there have been several efforts to determine the similarities and differences between murine neutrophils and PMN-MDSCs [23–26], further studies are still needed to accurately determine whether TANs, particular N2 neutrophils and PMN-MDSCs, represent two distinct or identical granulocytic cell populations. The recent work performed by Condamine *et al.* [27] might shed light on this issue. The authors have identified a marker, LOX1, that may distinguish PMN-MDSCs from neutrophils in the blood of patients with a variety of cancers. It should be emphasized that the assumption that all $CD11b^{+}Gr1^{+}$ cells in tumor-bearing mice and $CD66b^{+}CD15^{+}$ cells in humans are MDSCs should be avoided unless all required functional tests confirm that these cells are indeed suppressive [21].

Importantly, the most broadly used *in vitro* assays to study the suppressive activity of neutrophils or PMN-MDSCs isolated from advanced stages of murine tumors are based on measurements of the proliferation of naïve resting T cells (usually splenocytes) stimulated by anti-CD3/CD28 antibodies. However, the interaction of naïve T cells with granulocytic cell populations very likely does not reflect what actually transpires in tumors that are infiltrated with effector and memory T cells. In addition, it should be noted that many mouse and human studies use splenic $CD11b^{+}Ly\text{-}6G^{+}$ cells or circulating low-density $CD66b^{+}CD15^{+}$ cells as granulocytic MDSCs, respectively. The relevance of these cells to TANs and intratumoral immune suppression is not clear. For instance, there are several reports demonstrating that adoptively transferred tumor-specific effector T cells are resistant to intratumoral and systemic myeloid cell-induced suppression, and that these tumor-specific T cells can eradicate established tumors *in vivo* [28,29].

Another question to be addressed is the regulation of T-cell responses by neutrophils during the early stages of tumor development, where tumor-induced immunosuppressive mechanisms might not be fully developed. Thus, further studies are required to rigorously establish the regulation of antitumoral effector T-cell responses by TANs or PMN-MDSCs throughout all stages of tumor development.

Neutrophils are extremely dynamic and adaptable cells that are able to perform many different functions simultaneously. However, the majority of conclusions presented in the literature are weighted heavily toward one particular functional aspect of the neutrophil, while other functions are given less emphasis or remain untested. Hence, the concept of a predominantly protumoral role of neutrophils and their derived products in tumor biology may be drawn from a small slice of the total physiologic functions of neutrophils, leaving other equally important aspects to be overlooked. For example, the protumoral functions of neutrophil-derived proteases, including neutrophil elastase, have been attributed to their ability to degrade cytokines, chemokines, and their receptors; remodel the extracellular matrix; increase angiogenesis; and promote metastasis [30–32]. A study by Houghton and colleagues [33] showed neutrophil elastase uptake by lung adenocarcinoma cells, where it localized to endosomes and induced tumor cell proliferation by cleaving insulin receptor substrate-1, inducing hyperactivity of the phosphatidylinositol-3 kinase pathway and uncontrolled proliferation [33]. Also, neutrophil elastase release from TANs is able to potentiate antitumor immunity by increasing the susceptibility of tumor cells to tumor-specific cytotoxic T lymphocytes (CTLs). For instance, breast cancer cells exhibit a striking uptake of neutrophil elastase from the microenvironment, which yields increased expression of tumor-specific peptides on the cell surface and enhanced susceptibility to peptide-specific CTL lysis [34]. In addition, neutrophil elastase enhances antigen presentation by stabilizing and reducing membrane recycling of human leukocyte antigen class I molecules expressed on the surface of tumor cells and thus augments tumor-specific CTL [35]. Further confounding its purported protumoral role, neutrophil elastase can also inhibit tumor neoangiogenesis by activating antithrombin [36].

Another example is the multifaceted role of arginine metabolized by arginase-1 expressed in neutrophils. It is generally assumed that granulocyte-induced arginine deficiency in the tumor microenvironment induces suppression of T-cell responses and represents one of the mechanisms of tumor-mediated immune escape [26,37]. However, a recent study convincingly demonstrated that human $CD8^+$ T-cell antigen-specific cytotoxicity and perforin secretion are completely preserved in the absence of arginine, while antigen-specific proliferation as well as interferon-γ (IFN-γ) and granzyme B secretion are severely compromised [38]. Importantly, the production of the immunosuppressive cytokine interleukin-10 (IL-10) is also suppressed in an arginine-poor environment [39]. In addition, several studies indicated that arginine depletion might also result in tumor cell death, but only in those tumors that are auxotrophic for arginine, such as renal carcinoma, hepatocellular carcinoma, and melanoma [40,41].

Activated neutrophils can also release neutrophil extracellular traps (NETs) that are extracellular DNA structures composed of chromatin and neutrophil proteases [42]. To date, the bulk of the literature supports the notion that NETs exert strong protumoral effects during tumor progression by degrading the extracellular matrix and promoting cancer cell migration and metastatic colonization [43]. However, some data show potential antitumoral activity of NETs. For instance, several components of NETs, including myeloperoxidase and histones, have been shown to be cytotoxic to tumor and epithelial cells [44,45]. In addition, NETs are able to both activate dendritic cells and directly prime T cells by reducing their activation threshold [46,47].

For all these reasons, the current simplified scheme of classifying neutrophils as antitumoral and protumoral, which for many years has served as a satisfactory working hypothesis, might be inadequate today, especially for human tumors and TANs.

Caveats in Translating Findings from Mouse Tumor Models to Human Cancers

The power of mouse models has allowed us to generate many fundamental concepts in tumor immunology. However, it is important to keep an open mind about how closely these findings actually translate to human cancers. It is a sobering fact that the majority of cancer immune therapies that work well in mice fail to provide similar efficacy in humans. The average rate of successful translation from animal models to clinical cancer trials is less than 8% [48]. Here, we would like to detail some of the species-specific differences in tumor evolution, genetic heterogeneity, immune and inflammatory responses, and intrinsic biology of neutrophils that might have a profound impact on the tumor development and function of neutrophils in mouse tumors versus human cancers (Figure 1, Key Figure).

It is becoming apparent that human tumor development represents a process of microevolution with a broad range of selective pressures on multiple tumor subclones that harbor different driver and other mutations [49,50]. In addition to many intrinsic and tumor microenvironmental evolutionary pressures, prolonged selective immune pressures result in the development of adaptations that allow a tumor to escape any effective immune responses (Figure 1) since human neoplastic clones that are susceptible to the immune system will be killed. The remaining clones, which have survived sustained selective pressure by the host's antitumor immune response, will be increasingly resistant to adaptive immune system attacks and will gradually become the dominant population within the tumor. These surviving subclones will enter a final stage of tumor microevolution termed the 'escape phase', where protumoral mechanisms dominate and help to shape the fitness of these malignant cells (Figure 1) [10].

In contrast to human tumors, the majority of mouse tumor models use tumor cell lines originally derived from advanced tumors that have already been subjected to immune selection *in vivo*. These cell lines have been selected to grow rapidly *in vivo* and have thus already undergone extensive cancer immunoediting [10]. For this reason, the rapidly growing tumors in murine model systems do not accurately mirror the gradual stages of tumor development and the complex interactions between tumor cells and the tumor microenvironment that occur during the slow evolution of human tumors (Figure 1). Typically, mouse transplant models are characterized by a high tumor burden, minimal matrix, very rapid tumor growth, and the absence of important phases of cancer immunoediting such as elimination and equilibrium. Of note, the tumor-to-normal body ratio in many transplant mouse models can be several thousand times higher than that seen in humans. Furthermore, the fact that the mice are of the same inbred mouse strain means that the model will not reflect the huge human genetic diversity. In addition, most mouse tumors have many fewer mutations (and thus fewer neo-epitopes) than seen in many human cancers. All these features are dissimilar to the slow evolution of human cancers, specifically during the early stage of tumor development, and might have a profound impact on the function of inflammatory tumor-infiltrating cells, including neutrophils, in mouse tumors versus human tumors (Figure 1).

Genetically engineered mouse models with spontaneous tumor growth more closely reflect the slower multistage tumor development observed in humans. These mouse models are highly useful for evaluating the effects of specific mutation, deletion, or gene amplification of one or two genes during murine tumor progression [51]. However, they do not fully reproduce the genetic complexity of human tumors, where genes typically contain multiple point mutations and the specific gain or loss of genes varies enormously from one cell to another even within the

Key Figure

Fundamental Differences in Genetic Heterogeneity, Tumor Evolution, and Intrinsic Biology of Neutrophils Might Affect Neutrophil Phenotype and Function in Mouse versus Human Tumors.

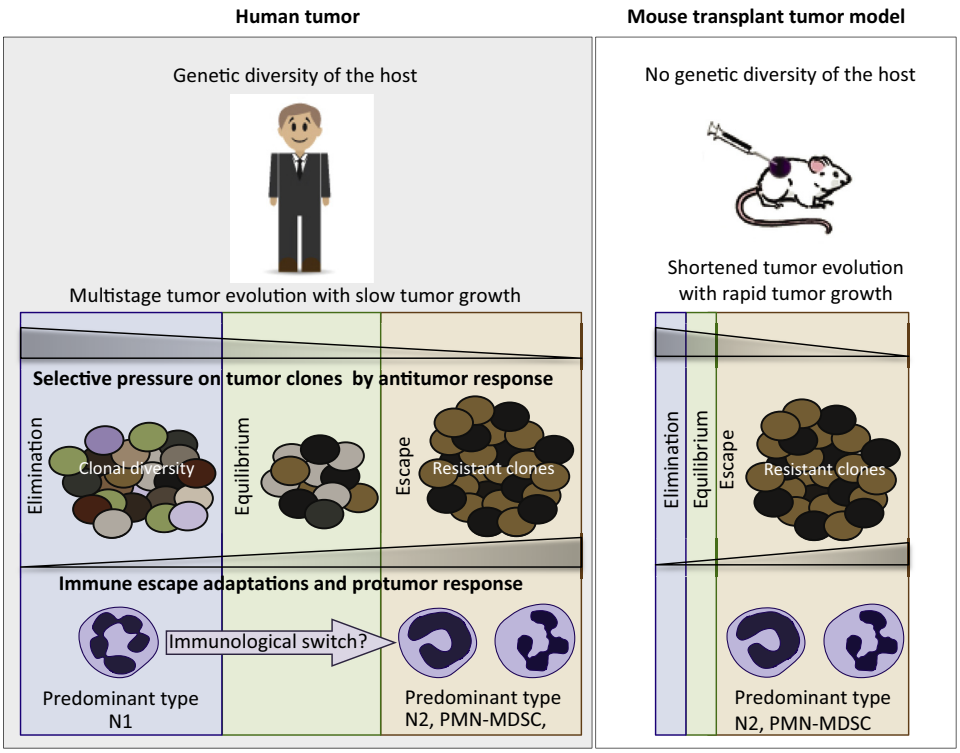

Figure 1. In contrast to humans, laboratory mice used for tumor models are inbred and genetically homogeneous. Moreover, they live in abnormally hygienic specific pathogen-free barrier facilities. Undoubtedly, this does not reflect relevant aspects of the human immune and inflammatory responses occurring during tumor development. Human tumors evolve slowly and undergo all steps of cancer immunoediting (elimination, equilibrium, and escape). At the first steps of the tumor evolution, tumor clones initiation, proliferation, and diversification occur along with selective pressure on these tumor clones by antitumor immune responses. Some clones may elicit immune responses and be killed, while other clones (brown and black color) may favor the immune tolerance and survive. At these stages, antitumoral neutrophils (N1) would be expected to develop. Consequent immune selection pressure allows surviving tumor clones to acquire sufficient adaptations that permit immune evasion. Subclones resistant to an immune response will gradually become predominant. Surviving clones form the third phase of immune escape, where protumor mechanisms predominate. At this stage, where antitumor response is weak against surviving clones, the accumulation of immunosuppressive N2 neutrophils and/or polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) might be expected. In contrast to humans, mouse tumor models use tumor cell lines that are originated from advanced tumors. These tumor cells have already undergone cancer immunoediting and thus are relatively resistant to an immune response. In addition, these tumor cell lines represent a homogenous tumor burden to the immune system that shortly enters the last phase of tumor evolution (escape) after transplantation in mice. This phase is mostly characterized by profound protumoral response with accumulation of immunosuppressive N2 neutrophils and/or PMN-MDSC.

same tumor [52]. In humans, premalignant clones with 'driver' and 'passenger' mutations coexist for prolonged periods of time and only selective pressure with antitumoral host responses will determine which carcinogenic mutations are neutral or advantageous. In genetic mouse tumor models, tumorigenesis is induced by engineered, previously characterized driver

mutations that normally result in growing a resistant clone. It is unlikely that tumors initiated by this resistant clone will be subjected to rigorously selective immune pressures and induce sustainable antitumor response. Because of a limited number of initiating driver mutations, mouse tumors are typically more homogeneous than human tumors. Therefore, although useful, genetic mouse models likely do not fully recapitulate the evolving interplay between the tumor microenvironment with neutrophils and malignant cells observed in humans. In addition, the slow generation time and insufficient amount of tumor tissue at early stages of development for analysis are other disadvantages of genetic mouse models in the study of TANs.

To date, both transplantable and spontaneous tumor models generate contradictory results regarding the *in vivo* role of neutrophils in tumor development. The majority of studies have shown that systemic depletion of neutrophils results in decreased tumor growth in mouse models, suggesting their protumoral role [11,33,53,54]. However, there are a number of papers demonstrating the opposite effect of neutrophils on tumor growth [55–58]. Moreover, there are conflicting data regarding neutrophil function even within the same type of tumor. For instance, some studies reported a protumorigenic role of neutrophils in lung cancer development in mice [59,60], whereas others demonstrated the opposite result [55,56].

Other potentially confounding factors that could dramatically alter tumor development and neutrophil behavior in mice versus humans are the substantial differences in the organization of the immune system that have been alarmingly emphasized in many recent reviews [48,61,62]. Importantly, these differences are even more dramatic between humans and the inbred specific pathogen-free laboratory mice used for tumor models [63]. Mouse and man use two different immunological defense strategies; in humans, immune resistance mechanisms dominate, while in mice tolerance mechanisms are highly favored [64]. Importantly, the use of inbred strains creates a wealth of homozygous recessive defects (many of which are still be discovered) that could skew the regulation of the immune response.

In addition to mouse-to-human differences in tumor evolution and immunity, there are significant intrinsic differences in the biology of neutrophils that might affect their function during tumor development in mice versus humans. In the following section, we highlight several of the known differences between human and mouse neutrophils.

(i) Neutrophils represent the majority of white blood cells in humans (50–70%), but are less common in mice (10–30%) [61].
(ii) In mice, Gr-1 and Ly-6G are well-established markers to identify granulocytes. In humans, granulocytes do not express the Gr-1 and Ly-6G antigens, making the direct comparison of murine and human granulocytes impossible.
(iii) The structure and physicochemical properties of key molecules such as selectins, phosphatidylinositol-3 kinases, and serine proteases are different, which might affect the migration of neutrophils into tumor sites, intracellular cell signaling, and neutrophil effector mechanisms [65–67].
(iv) Mice do not express FcαRI, one of the most powerful Fc receptors that triggers effector functions such as oxidative burst, cytokine release, NETosis, and phagocytosis in neutrophils as well as sufficient ADCC [68,69].
(v) The granule content in human neutrophils is very different than in mice, which might dramatically alter neutrophil effector functions in mice versus humans. For instance, mouse neutrophils do not produce defensins, while human neutrophils do [70]. Moreover, the expression of bactericidal/permeability-increasing protein, myeloperoxidase, β-glucuronidase, lysozyme, alkaline phosphatase, and arginase-1 is much higher in humans than in mice [71].
(vi) The production of immunoregulatory cytokines also differs. For example, secretion of IL-10 is well established in murine neutrophils, while human neutrophils appear not to

secrete detectable amounts of IL-10 [72]. In addition, whether human neutrophils can express IL-6, IL-17A, IL-17F, and IFN-γ like their mouse counterparts is the subject of conflicting reports [73].

(vii) The activation pathways of reactive oxygen species (ROS) production differ between human and mouse neutrophils [74].

(viii) Many chemokines and their receptors, including those that affect neutrophil trafficking, are absent in mice. The chemokines IL-8 (CXCL8) and its receptor CXCR1, neutrophil-activating peptide-2 (CXCL7), IFN-inducible T-cell chemoattractant (CXCL11), monocyte chemoattractant protein-4 (CCL13), HCC-1 (CCL14), hemofiltrate CC chemokines-2 (CCL15), pulmonary and activation-regulated chemokine (CCL18), myeloid progenitor inhibitory factor-1 (CCL23), and eotaxin-2/3 (CCL24/CCL26) have all been identified in humans but not in mice [75]. Thus, targeting CXCR2 to modulate the recruitment of neutrophils in murine tumors might not represent a reliable system to study neutrophils in humans.

(ix) The delayed-type hypersensitivity reaction in mice tends to be more neutrophil rich after the antigen challenge, despite the fact that murine peripheral blood has a relative paucity of neutrophils compared with humans. In humans, delayed-type hypersensitivity is characterized by a rapid neutrophil response, followed by a dramatic influx of mononuclear cells, T cells, and macrophages [61,76].

(x) The mechanisms and relative importance of various modes of immunosuppression (e.g., nitric oxide, ROS, arginase-1) are different between humans and mice. In contrast to murine neutrophils, arginase-I is constitutively expressed in human granulocytes and is not modulated by a variety of proinflammatory and anti-inflammatory stimuli *in vitro* [77]. Human arginase-I is localized and well-preserved in azurophil granules and it appears that human neutrophils do not significantly metabolize extracellular arginine in the milieu [77]. Thus, the arginine-dependent suppression of T-cell responses observed in mouse tumor models might not take place in humans.

Therefore, for the multiple reasons outlined here it is important to consider the possibility that the neutrophil response in mouse tumor models may not occur in precisely the same way as it does in humans.

Challenges in the Study of Human TANs

Murine experimental studies have highlighted opposing roles of neutrophils in cancer, however there is no direct evidence yet that neutrophils tend to be protumoral in human tumor tissue and it is still unproven whether N1/N2 polarization of tumor-infiltrating neutrophils occurs in humans. The majority of experimental approaches to study neutrophils in cancer patients have relied on circulating blood neutrophils and make the assumption that these cells have a similar function in the tumor. Results of these studies have been recently summarized and reviewed [8,78]. Unfortunately, there is virtually no functional data relating to TANs in humans. The primary challenges that have limited progress in this field in humans include technical difficulties in obtaining fresh tumors, inefficient digestion of human tumor tissue, failure to isolate TANs while preserving cell surface markers and functionality, and the lack of cognate mouse myeloid-cell markers in humans. The logistical, ethical, and regulatory difficulties in obtaining human tumor tissue for research also act to discourage such studies.

Given all these obstacles, the characterization of neutrophils within human tumor tissue has been mostly limited to detection of only single or double granulocytic markers by immunohistochemistry and correlations of neutrophil counts to clinical prognosis. Intratumoral accumulation of neutrophils is associated with poor prognosis for patients with head and neck cancer [79], renal cell carcinoma [3], hepatocellular cancer [80], and some other types of cancers [81]. By contrast, a high neutrophil count has been associated with a favorable prognosis in patients with gastric cancer [82]. The results in lung cancer have been mixed. In one recent study, the presence of TANs in nonsmall cell lung cancer did not correlate with overall survival [1]. In

another study, an increased intratumoral neutrophil-to-CD8 T-cell ratio was a poor prognostic factor [2]. Similarly, contradicting results were reported for colorectal cancer [83,84]. Importantly, there have been no reports directly demonstrating a protumoral role of TANs in any type of human tumors.

There are several explanations as to why the density of TANs is an inconsistent prognostic factor for the clinical outcome of cancer patients. First, all clinical studies have used immunohistochemical analyses of tumor tissues to correlate the presence of granulocytes with prognosis. The limitations of this type of analysis prevent comprehensive descriptions of the neutrophil phenotype, as well as evaluation of the functional state of these cells in the tumor. The heterogeneous expression of many surface receptors on TANs suggests that different subpopulations of TANs exist in the tumor and might perform different functions [27,85,86]. Thus, the variation in the accumulation of different subsets of neutrophils in tumor tissue might explain why the percent of total neutrophils in the tumor is an inconsistent predictor for survival of cancer patients. Second, an immunogenic 'switch' from antitumor to protumor phenotype might take place [87]. Specifically, TANs might lose or change their functions as the tumors progress [85,86]. Accordingly, a simple neutrophil count in tumor tissue at any one time point (where the protumor vs. antitumor status of the neutrophils is not known) may not be an accurate parameter for clinical prognosis.

To study human TAN function, the generation of high-quality single-cell suspensions from human tumor tissues is required. However, to date no standardized techniques exist for the disaggregation of human tumor tissue. Currently available methods may cause alterations in the true immune cell profile and thus can provide misleading results. We have conducted a study in which we critically evaluated current techniques used to prepare human tumors for immunologic studies [88] and found that many of these approaches use an unbalanced composition of enzymes that artificially cleave multiple cell-surface markers. This digestion-induced effect might lead to false conclusions about the presence of specific cellular populations and their biologic characteristics. To overcome this technical difficulty, we optimized a disaggregation method designed for human lung tumors that preserves the phenotype and function of the immune cells, including neutrophils [85,88]. Our data suggest that a similar approach should be applied to any type of human tumors to be analyzed, as conditions vary depending on tumor type.

Given that human tumor development represents an ongoing process of evolution, it is critical to understand the complex interaction of TANs with the tumor microenvironment during all stages of tumor growth. One of the major challenges in TAN biology is deciphering the complex interaction of activated neutrophils with T cells in the tumor microenvironment. Understanding the role of TANs in the regulation of the T-cell response is especially important because CTLs are the major effector cells mediating antigen-driven antitumor immunity [89]. There is increasing evidence that activated neutrophils can interact with T cells in dichotomous ways. Several studies have shown that neutrophils can present antigens and provide accessory signals for T-cell activation [90,91]. Other studies have demonstrated that peripheral blood neutrophils can suppress antigen-nonspecific T-cell proliferation through the depletion of arginine and the production of ROS [92,93]. To date, the suppressive function of granulocytic cells in cancer patients has generally been attributed to a circulating low-density PMN-MDSC population [94]. However, there has been some uncertainty about whether PMN-MDSCs are present in human tumors and whether the majority of TANs are actually PMN-MDSCs.

We have begun initial studies with the goal of phenotypic and functional characterization of human TANs at early stages of tumor development. Although the presence of a minor suppressive subpopulation of TANs cannot be excluded, our data suggest that in patients

with early stage lung cancer, TANs do not significantly contribute to the inhibition of T-cell responses [85]. In fact, the TANs isolated from the vast majority of small-sized, early stage tumors were actually able to stimulate T-cell responses to varying degrees. We found that direct cell–cell contact was important for the neutrophil-mediated stimulation of T-cell proliferation. An important feature of this interaction was crosstalk between TAN and T cells resulting in mutual activation. During their interaction, T cells further upregulated their activation markers and produced more IFN-γ, whereas TANs increased their survival time and expressed the co-stimulatory molecules CD86, CD40, CD54, OX40L, and 4-1BBL.

Our data are consistent with some literature showing the antitumor potential of neutrophils during tumor growth in mouse models [95,96]. For instance, Suttmann and colleagues [19] demonstrated that polymorphonuclear neutrophils were an indispensable subset of immunoregulatory cells and orchestrated T-cell chemotaxis to the bladder during Bacillus Calmette–Guérin immunotherapy. Augmentation of T cell proliferation and/or survival by tumor-infiltrating neutrophils was found to be critical in the establishment of antitumor immunity following photodynamic therapy [18]. Liu *et al.* [55] have also demonstrated that neutrophils could control early tumorigenesis in lung by direct cytotoxic effects.

We also identified a specialized subpopulation of TANs with 'hybrid' characteristics of neutrophils and antigen-presenting cells [86]. These antigen-presenting cell-like hybrid TANs accumulated only in small-sized, early stage tumors and dramatically contributed to the strong T-cell stimulatory effect of all TANs observed in small tumors. Based on the observation that TANs lost their T cell stimulatory ability in large tumors, we postulate that this antitumor TAN response could represent the footprint of incomplete or ongoing elimination or equilibrium phases of immunoediting during the early stages of lung cancer. It appears that the antitumoral potential of neutrophils diminishes during tumor progression, supporting the concept of an immunogenic 'switch' from antitumor to protumor phenotype during tumor progression (Figure 1) [87,97]. By contrast, these T cell-stimulatory TANs secrete factors such as macrophage inflammatory protein-1α, fibroblast growth factor, hepatocyte growth factor, and epidermal growth factor that could be protumorigenic and act as growth and survival factors for tumor cells, as well as support angiogenesis [85,98]. Thus, it is difficult to accurately determine if the TANs in early stages of human tumors are actually N1 or N2 neutrophils.

We can also speculate that although small-sized early stage tumors in humans have already reached a considerable size and represent an escape stage of immunoediting process, the process of tumor evolution is still ongoing, because the profound immunosuppressive environment is not fully developed yet and antitumoral neutrophils still prevail in small-sized tumors (early escape stage). The resistant tumor clones, which survived the immunoediting process, likely further develop immunosuppressive mechanisms to sustain tumor growth into more advanced stages. At these advanced tumor stages, the suppressive environment appears to disable antitumoral N1 neutrophils and perhaps even convert them into the protumoral N2 type (late escape stage). Thus, it is very possible that N2 protumoral TANs represent the consequences of tumor progression, but not a cause. This hypothesis has not been experimentally confirmed, but we are trying to test it with TANs from patients with advanced lung cancer (Stages III and IV). This has turned out to be logistically challenging since these individuals do not routinely undergo tumor resection and are managed with chemotherapy and radiation therapy.

Concluding Remarks

The goal of this review is to raise awareness about some key issues relating to extrapolating data from mouse tumor models to human cancers. It is becoming increasingly obvious that critical species-specific differences in tumor evolution and immunity, as well as significant

Outstanding Questions

Does the antitumoral N1/protumoral N2 paradigm that appears to exist in mice occur in human cancer?

Are these N1/N2 neutrophils truly bona fide neutrophil subtypes or do they represent a continuum of activation and maturation states?

Is there the immunogenic 'switch' from antitumor to protumor neutrophil phenotype as tumors progress from early stages to advanced stages?

Can the neutrophil response observed in rapid growing murine tumors be extrapolated to human cancers that evolve very slowly?

Can antitumor functions of TANs be harnessed to treat cancer?

species differences in the biology of neutrophils create challenges and risks in translating data derived from mouse studies into humans. Moreover, there is a lack of data about the function of neutrophils in human tumors. Thus, to date, it is very difficult to reliably compare the phenotypes and functions of neutrophils during tumor progression in mice versus humans.

Since human tumor development represents a process of gradual evolution, it is critical to understand the complex interaction of TANs with the tumor microenvironment at all stages of tumor evolution, as their relationship appears to change over time. Although the first attempts have been made, there is a continuing need to develop new and innovative approaches to characterize granulocytes and their subpopulation in human cancers and to characterize the variety of their functions in the human tumor microenvironment (see Outstanding Questions). This knowledge will allow us to develop different therapeutic strategies to regulate the function of TANs depending on tumor stage. It is hoped that understanding how to direct and maintain the human TANs toward antitumor effector cells will open new therapeutic options in the future design of active immunotherapy to potentially boost natural or vaccine-induced antitumor immunity.

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Phenotype and function of tumor-associated neutrophils and their subsets in early-stage human lung cancer

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FOCUSSED RESEARCH REVIEW

Phenotype and function of tumor-associated neutrophils and their subsets in early-stage human lung cancer

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Abstract Neutrophils accumulate in many types of human and murine tumors and represent a significant portion of tumor-infiltrating myeloid cells. Our current understanding of the role of neutrophils in tumor development has depended primarily on murine models of cancer. However, there are crucial species differences in the evolution of tumors, genetic diversity, immune and inflammatory responses, and intrinsic biology of neutrophils that might have a profound impact on the tumor development and function of neutrophils in mouse versus human tumors. To date, the majority of experimental approaches to study neutrophils in cancer patients have been limited to the examination of circulating blood neutrophils. The phenotype and function of tumor-associated neutrophils (TANs) in humans, particularly in the early stages of tumor development, have not been extensively investigated. Thus, the long-term goal of our work has been to characterize human TANs and determine their specific role in tumor development. Here, we summarize our findings on human TANs obtained from human early stage lung cancer patients. We will describe the phenotypes of different TAN subsets identified in early stage lung tumors, as well as their functional dialog with T cells.

Keywords Human lung cancer · Human tumor microenvironment · Tumor-associated neutrophils · Regulatory myeloid suppressor cells · Neutrophil and T-cell interaction

Abbreviations

EGF	Epidermal growth factor
FGF	Fibroblast growth factors
G-MDSCs	Granulocytic-myeloid-derived suppressor cells
HGF	Hepatocyte growth factor
ICAM-1	Intercellular adhesion molecule 1
LDN	Low-density neutrophils
MCP-1	Monocyte chemotactic protein 1
MPO	Myeloperoxidase
NDN	Normal density neutrophils
NSCLC	Non-small cell lung carcinoma
NY-ESO-1	New York-esophageal cancer-1
PBNs	Peripheral blood neutrophils
PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cells
TANs	Tumor-associated neutrophils
VEGF	Vascular endothelial growth factor

Introduction

Neutrophils accumulate in many types of human and murine tumors and regulate nearly all steps of tumor progression [1–4]. It is becoming apparent that neutrophils are able to shape and regulate immune and inflammatory responses against tumor cells. Our current understanding of

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neutrophil function in tumor progression has been inferred from various types of mouse tumor models. These mouse models suggest that neutrophils can exert both pro-tumor and anti-tumor effects on tumor development [1, 5]. Given these varied effects of neutrophils, the concept of neutrophil diversity and plasticity has begun to emerge in murine tumor models, leading to the paradigm of anti-tumoral "N1 neutrophils" versus pro-tumoral "N2 neutrophils" proposed by Fridlender and colleagues [5]. Currently, a pro-tumoral and immunosuppressive role of neutrophils in transplanted mouse tumor has become the dominant view in the field.

It should be noted that there are substantial species differences in tumor progression, host genetic diversity, immune and inflammatory responses, and the intrinsic biology of neutrophils that likely have a profound impact on both tumor development and neutrophil function in mice versus humans [6, 7]. In contrast to human tumors, the majority of mouse tumor models use tumor cell lines that have been derived from spontaneously arising advanced tumors that have already been subjected to immune selection in vivo. These cell lines have been selected to grow rapidly in vivo (usually in the flanks) and have thus already undergone cancer immunoediting and "Darwinian" selection [8]. Hence, the majority of mouse tumor models lack prolonged the initial phases of multistage tumor evolution, such as elimination and equilibrium phases that would be expected to occur in humans. Data obtained from these transplantable mouse models mostly reflect the immune response as it occurs during the advanced stage of tumor development, at which time pro-tumoral mechanisms already prevail. At this stage, the accumulation of immunosuppressive N2 neutrophils and/or polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) might be expected. In contrast, human tumors are slower to evolve. During the first steps of tumor evolution in humans, the processes of tumor clone initiation, proliferation, and diversification occur concomitantly with selective pressure on tumor clones by anti-tumoral immune responses [9]. Most clones likely elicit an immune response and are killed, while other clones mediate immune tolerance and survive during the sustained selective pressure by the host's anti-tumor immune response. At these early stages of tumor evolution, anti-tumoral neutrophils (N1) would be expected to predominate. Surviving clones will be increasingly resistant to the adaptive immune system's attacks and will gradually come to dominate within the tumor. These early stage human tumors have entered the escape phase of the immunoediting process and appear to develop an increasingly immunosuppressive environment. The functional state of TANs at these early stages of tumor development has not been well studied yet. As the tumor progresses, the composition of the tumor microenvironment and its cytokine milieu likely become more suppressive for the anti-tumor host responses. Thus, it is very possible that human and murine neutrophils represent cells with different functional states that exist during the early and advanced stages of tumor evolution.

To date, data on the functional role of neutrophils in human cancer are still relatively scarce and have largely been obtained from experiments relying on circulating peripheral blood neutrophils. A recent summary of these studies has described neutrophils as versatile and heterogeneous cells that exert various effects on T-cell migration, activation, differentiation, and effector functions [10–12]. It has been suggested that one way to segregate and classify these heterogeneous populations of neutrophils is by their sedimentation properties in density gradients: i.e., normal density neutrophils (NDNs) versus low-density neutrophils (LDNs) [11, 13]. However, even these LDN and NDN groups contain diverse cell populations with different functions. LDNs contain immature neutrophils and activated mature neutrophils that perform immunosuppressive and pro-inflammatory functions [11, 13]. The T-cell immunosuppressive LDNs are also known as granulocytic-myeloid-derived suppressor cells (G-MDSCs) [14]. NDNs mainly consist of resting neutrophils. However, under certain disease conditions, smaller subsets of immature neutrophils (mostly band cells) and activated mature neutrophils with different immunoregulatory effects toward T cells have also been detected [11, 15, 16]. The precise phenotypes and functions of all these neutrophil subpopulations within LDNs and NDNs still require further examination. Thus, although the concept of neutrophil diversity and plasticity has begun to emerge in both human and mouse tumor models, uncertainty regarding the phenotypes, functional roles, and relationships between different granulocytic cell populations during tumor progression persists.

As mentioned above, the majority of experimental approaches to studying neutrophils in cancer patients rely on circulating peripheral blood neutrophils (PBNs) and thus make the assumption that peripheral neutrophils function similarly to those found within the tumor. This assumption may not be valid, since the phenotype and function of tumor-associated neutrophils (TANs) in human subjects, particularly in the early stages of tumor development, have not been extensively investigated. Importantly, in contrast to PBNs, the interplay between TANs and the tumor microenvironment likely causes phenotypic and functional changes in TAN populations during tumor progression. For this reason, to understand the role of neutrophils during human tumor progression, TANs, themselves, should be investigated and compared with PBNs. The primary challenges that have limited progress in this area include technical difficulties in obtaining fresh human tumors, inefficient digestion of human tumor tissue, failure to isolate TANs while preserving cell surface markers and functionality, the

fragility of human TANs (i.e. they do not survive freezing and thawing), and the lack of cognate mouse myeloid-cell markers in humans. In contrast to PBNs, isolation of TANs from tumors requires a more prolonged multi-step procedure, where technical issues might have some effect on the phenotype and function of TANs. The logistical, ethical, and regulatory difficulties in obtaining human tumor tissue for research also act to discourage such studies.

By having a close collaboration between the surgeon and the laboratory, we have overcome these difficulties and have begun the initial studies with the goal of phenotypic and functional characterization of human TANs. Here, we will summarize and discuss our latest findings regarding the TANs and their role in the regulation of the T-cell responses in patients with early stage lung cancer. Understanding the role of TANs in regulating T-cell responses in cancer patients is particularly important, because cytotoxic T lymphocytes are the chief effector cells mediating antigen-driven anti-tumor immunity.

Optimized disaggregation of tumor tissue is the first critical step in studying human TANs

Generation of high-quality single-cell suspensions from human tumor tissues is required to study human TAN function. To date, specific disaggregation techniques that are tailored to specific types of human tumors have not been developed. Rather, a wide variety of enzymes have been used to digest solid tumors, under the assumption that almost any particular enzymatic dissociation technique will provide high cell yield without affecting the functional activity of the cell populations under study. In general, methods of enzymatic digestion which produce higher cell yields are more harsh and tend to induce more artifacts through the cleavage of cell surface markers [17, 18]. Without careful assessment of the effects of enzymatic digestion on phenotype, a particular method may cause alterations in the true immune cell profile and thus provide misleading results. We have conducted a study in which we critically evaluated the current techniques available in the literature used to prepare human tumors for immunologic studies and found that many approaches used an unbalanced composition of enzymes that inadvertently cleaved multiple cell surface markers [18]. Such digestion–induced effects might lead to false conclusions about the presence or absence of specific cellular populations and their biologic characteristics. Our group investigates the tumor microenvironment of human lung cancer, so we rigorously tested and validated various techniques to process human non-small cell lung carcinoma (NSCLC) specimens. Our objective was to balance high immune cell yield and high cell viability with maintenance of key surface markers and functional characteristics. Our final approach to prepare human lung tumors used a combination of non-traumatic, gentle mechanical manipulation, and an optimized cocktail of specific enzymes used at low doses. We have established that this disaggregation approach optimized cell yield and cell viability, retrieved all major tumor-associated cell populations, and maintained the expression of cell surface markers for both lineage definition and in vivo effector functions [18]. Using this methodology, we have been able to develop a complete phenotypical and functional description of TANs [19, 20].

Characteristics of TANs in early stage human lung cancer

In mice, antibodies to the CD11b and Ly-6G antigens are well-established tools to identify granulocytes. In humans, granulocytes do not express the Ly-6G antigen, making the direct comparison of murine and human granulocytes impossible. To date, the characterization of neutrophils within human tumor tissue has largely been limited to the detection of only single or double granulocytic markers [for example, CD15, CD66b or myeloperoxidase (MPO)] by immunohistochemistry. However, there had been no reports that extensively evaluated the phenotype of TANs in human tumors. Thus, the first goal of our investigation was to develop a complete phenotypical description of TANs in humans. For this purpose, we used fresh, surgically resected early stage lung tumor tissue.

We performed an extensive phenotypic analysis of tumor-associated neutrophils in high-quality, single-cell suspensions obtained from fresh NSCLCs using our optimized disaggregation method. In multicolor flow cytometry, TANs could be defined as $CD11b^{+}CD15^{hi}CD66b^{+}MPO^{hi}Arg1^{+}CD16^{int}IL\text{-}5Ra^{-}$ cells and were found in varying frequency, ranging from 2 to 20% of live cells in the tumor microenvironment [19]. We showed that neutrophils recruited into lung tumors exhibited an activated phenotype when compared with circulating peripheral blood neutrophils (Fig. 1) [19]. For example, recruited TANs expressed the "classic" activation markers characterized by up-regulation of the adhesion molecule CD54 (ICAM-1) and down-regulation of CD62L (L-selectin), CXCR1, CXCR2, and CD16 [21]. CD54 plays an important role in cellular adhesion, endothelial transmigration, and stabilizing cell–cell interactions. Recent studies have shown that CD54 can also be actively involved in the neutrophil-dependent potentiation of IL-12 and IFN-γ release by DCs and NK cells, respectively [22]. Of note, CD54 (ICAM-1) was found to be an N1 neutrophil marker in murine tumor models [5].

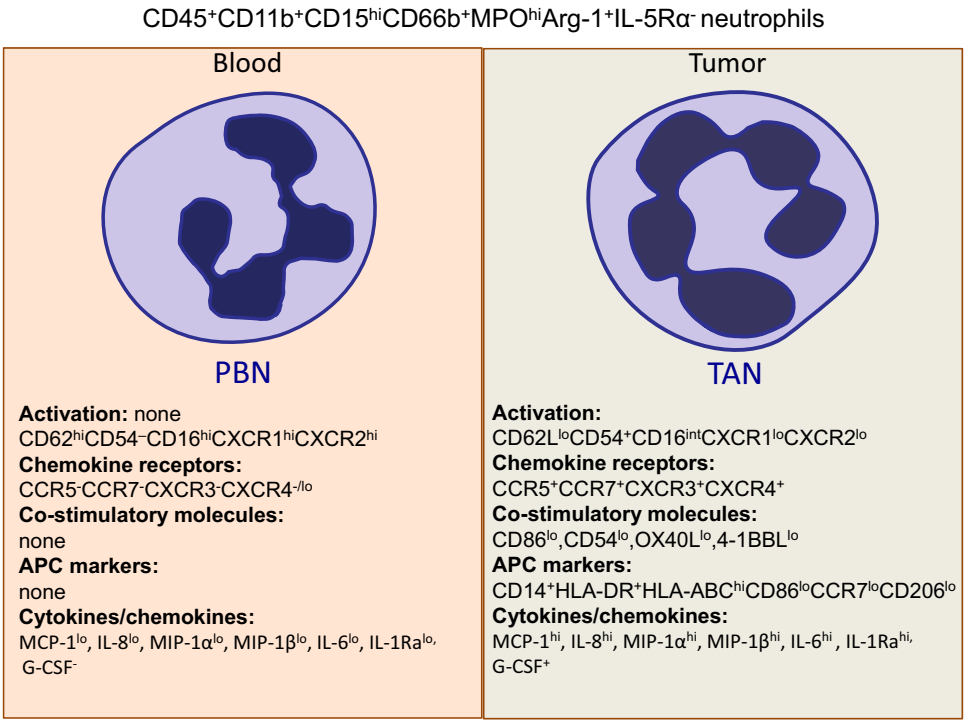

Fig. 1 Phenotypic characteristic of peripheral blood neutrophils (PBNs) and tumor-associated neutrophils (TANs) in early stage human lung cancer. Both PBNs and TANs express the canonical neutrophil markers $CD45^{+}CD11b^{+}CD15^{hi}CD66b^{+}MPO^{hi}Arg\text{-}1^{+}IL\text{-}5R\alpha^{-}$. In addition to canonical neutrophil phenotype, TANs express an activated phenotype characterized by up-regulation of the adhesion molecule CD54 and down-regulation of CD62L (L-selectin), CXCR1, CXCR2, and CD16. TANs up-regulate co-stimulatory molecules, which acquire a novel repertoire of chemokine receptors and a new cytokine/chemokine profile. In some small-sized, early stage tumors that produce IFN-γ and GM-CSF, TANs could differentiate into a subset of cells exhibiting a partial phenotype of professional APCs ($CD14^{+}HLA\text{-}DR^{+}HLA\text{-}ABC^{hi}CCR7^{low}CD86^{low}CD206^{low}$)

In addition to changes in activation, we also found that activated TANs expressed a new chemokine receptor profile in the tumor microenvironment (Fig. 1). We observed that TANs up-regulate CCR5, CCR7, CXCR3, and CXCR4, and down-regulate CXCR1 and CXCR2, compared to peripheral blood neutrophils in lung cancer patients [19]. Interestingly, the expression of CCR5 and CXCR1 was modulated on all TANs, whereas the changes in CCR7, CXCR3, and CXCR4 expression were seen only on subsets of TANs. The elevated expression of CCR1, CCR2, CCR3, CCR5, CXCR3, and CXCR4 on neutrophils has also been reported for cells directly isolated from bronchoalveolar lavage fluid of patients with chronic inflammatory lung diseases and from synovial fluid of patients with rheumatoid arthritis [23]. It appears that, in addition to playing a role in neutrophil recruitment, these newly expressed chemokine receptors, which are up-regulated in neutrophils at sites of inflammation, can also regulate many other neutrophil functions. For instance, the binding of CXCL11 to CXCR3 stimulated the release of α-defensin and induced strong bacterial cytotoxicity by neutrophils, whereas the binding of CXCL12 to CXCR4 reduced the respiratory burst in pulmonary neutrophils [23]. Furthermore, a recent study found that CCR7 is involved in the migration of murine neutrophils to lymph nodes, where they may induce and/or modulate adaptive immune responses [24]. Finally, the high expression of CCR5 on neutrophils has been found to play a role in sequestering CCR5 ligands during the resolution of inflammation in murine peritonitis [25].

TANs also express co-stimulatory molecules such as CD86, CD54, OX40L, and 4-1BBL at low levels; however, these are dramatically up-regulated during interactions with activated T cells (Figs. 1, 2) [19, 20]. Normally, resting neutrophils do not express these co-stimulatory molecules on the cell surface, but rather store them in cytoplasmic granules [26]. During the activation process, neutrophils are able to transfer these stored molecules onto the surface and then synthesize them de novo [26]. Surprisingly, in some early stage lung cancer patients, we identified a subset of activated TANs with atypical expression of surface markers which normally belong to the professional antigen-presenting cells (APCs) [20]. This subset of TANs displayed a combination of canonical neutrophil markers (CD11b/CD66b/CD15) and APC markers (CD14/HLA-DR/CCR7/CD86). Given this unique hybrid phenotype, we termed this subset ‘‘APC-like hybrid TANs’’.

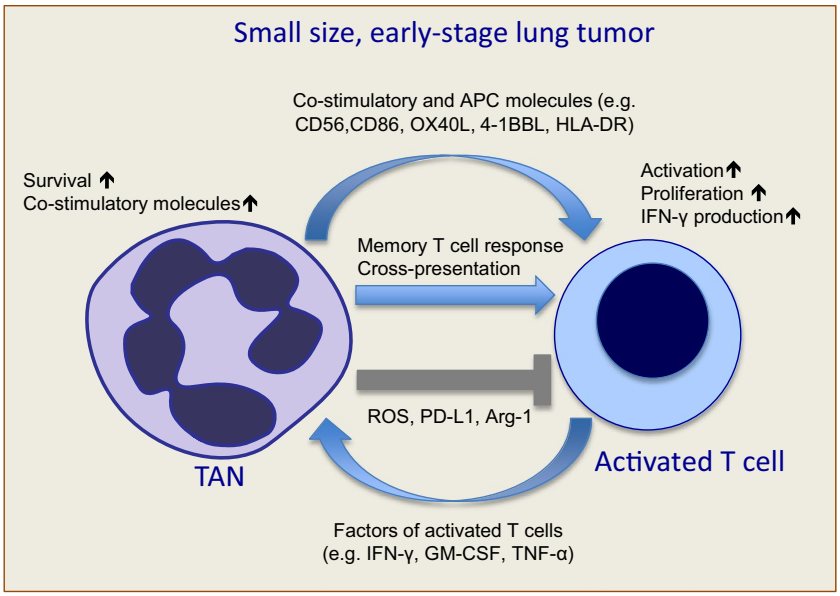

Fig. 2 Cross talk between TANs and activated T cells in small, early stage human lung cancers. IHC staining of lung tumor sections for TANs and T cells reveals frequent contact between these cells, suggesting that they may interact in the tumor microenvironment of lung cancer patients. This interaction of TANs with activated T cells results in mutual cell activation. Activated T cells produce pro-inflammatory factors (e.g., IFN-γ, GM-CSF, and TNF-α) that increase the activation of TANs, their survival time, and up-regulate co-stimulatory molecules. TAN-affected activated T cells, in turn, further up-regulate their activation markers, produce more IFN-γ, and proliferate. In some patients, TANs can acquire new characteristics of "professional APCs" and become APC-like hybrid TANs. These APC-like hybrid TANs are able to directly stimulate antigen-specific autologous memory and effector T-cell responses, as well as cross-present tumor antigens bound with specific antibodies. It seems that neutrophil-mediated suppression of T-cell response through the ROS, PD-L1, and arginine depletion mechanisms is not active in early stages of tumor development, but might be turned on at more advanced stages

The frequency of this hybrid population declined as tumors grew larger and they were almost completely absent in large (but still early stage) tumors. Importantly, in addition to a unique phenotype, these hybrid TANs also acquired new functions compared with canonical TANs and PBNs, which will be discussed further below.

Neutrophils with a composite phenotype of APCs, particularly those with the same phenotype as dendritic cells, have recently been described in many non-cancerous inflammatory conditions and were termed ''neutrophil-DC hybrids'' [27–30]. However, the APC-like hybrid TANs which we have identified in early stage human lung tumors are not quite similar to these "neutrophil-DC hybrid" cells. We have found that neutrophils in some early stage lung tumors do not exhibit the full phenotype of dendritic cells, but rather acquire only a partial dendritic cell phenotype, as well as a partial monocyte/macrophage phenotype. The APC-like hybrid TANs do not express many other macrophage- and DC-lineage defining markers such as CD209, CD204, CD83, CD80, CD1c, CD163, and CCR6 [20]. Thus, the differentiation of neutrophils in early stage lung tumors results in the formation of activated canonical TANs ($CD11b^{+}CD66b^{+}CD15^{hi}HLA\text{-}DR^{-}CD14^{-}$), as well as a unique rare subset of TANs with composite characteristics of neutrophils and APCs ($CD11b^{+}CD66b^{+}CD15^{hi}HLA\text{-}DR^{+}CD14^{+}$).

In addition to the phenotypic changes observed in TANs, the tumor microenvironment can also prolong the survival of recruited short-lived circulating neutrophils. We found that TANs were able to survive in cell culture longer than circulating neutrophils. In the presence of tumor-conditioned media in vitro, both TAN and naïve blood neutrophils exhibited prolonged survival [19]. This effect is likely due to the fact that pro-inflammatory factors (such as IFN-γ, IL-6, IL-8 and GM-CSF) present in tumor-conditioned media are known to prolong the lifespan of human neutrophils in vitro by delaying apoptosis [19, 31, 32]. For instance, we found that TANs are able to produce a large amount of IL-8 as compared to PBNs [19]. This TAN-derived IL-8 can increase neutrophil survival as well as recruit more neutrophils.

In turn, the cytokines and chemokines produced by TAN subsets within the lung tumor are critical in mediating their effects on the tumor microenvironment and the local inflammatory processes. We found that, in contrast to circulating neutrophils, TANs produce a variety of pro-inflammatory mediators such as CCL2 (MCP-1), CCL3 (MIP-1α), and IL-6, as well as anti-inflammatory IL-1Ra

[19]. MCP-1 is a classic monocyte chemoattractant that can also promote adaptive immune changes, particularly Th1-type responses [33]. TANs also secrete CCL3 (MIP-1α). The role of neutrophil-derived CCL3 is critical in the recruitment of immature DCs to sites of inflammation, as well as in the initiation of a protective Th1 response [34]. The cross talk that occurs between neutrophils and dendritic cells has been described previously [35]. As a result of this interaction, neutrophil-activated DCs produce the pro-inflammatory cytokine IL-12, which induces T-cell proliferation. As we often see co-localization of TANs with HLA-DR$^+$ APCs in lung tumors [19, 20], we infer that TANs create a favorable environment for T-cell activation and differentiation at the early stages of tumor development. On the other hand, TAN-secreted MIP-1α may act as a growth, survival, and chemotactic factor for tumor cells and thus could be considered pro-tumorigenic [36]. In our study, we did not see high levels of pro-angiogenic VEGF, but there were other growth factors that might support angiogenesis, such as FGF, HGF, and EGF. Thus, these data suggest a complex role for TANs in early stage human lung cancer.

Dialog between TAN and T cells in early stage lung cancer

Given that cytotoxic T cells are the major effector cells mediating anti-tumor immunity, there is a great deal of emphasis placed on understanding the regulation of T-cell responses by tumor-infiltrating myeloid cells, including neutrophils. In general, neutrophils are extremely versatile cells and depending on environmental cues may exert diverse effects on T-cell response. Numerous studies have convincingly demonstrated that activated neutrophils are able to stimulate T-cell responses by providing co-stimulatory signals [16, 37, 38]. Moreover, in some circumstances, activated neutrophils can even present antigens and thus function as professional APCs [38–40]. Neutrophils can also exert inhibitory effects on T-cell responses via the production of reactive oxygen species and the depletion of extracellular arginine, suggesting a degree of effector function plasticity [15, 41, 42].

These studies, however, have only evaluated peripheral neutrophils and have not examined the interaction of TANs with T lymphocytes in the human tumor microenvironment. In cancer, immunosuppressive function of neutrophils is often associated with a population of circulating low-density granulocytes termed PMN-MDSC [14]. However, there has been some uncertainty whether PMN-MDSC are present in human tumors, particularly at early stages of development, and whether the majority of TANs are actually PMN-MDSC. Given the dual functionality of neutrophils in the regulation of T-cell responses, one of our goals was to determine whether lung tumors at an early stage of development can convert recruited granulocytes into cells with T-cell suppressive activity or whether neutrophils represent a part of anti-tumoral host response with T-cell stimulatory activity.

First, we found the frequent co-localization of TANs and T cells in tumor tissue in lung cancer patients, suggesting that TANs can functionally interact with T cells [19]. Although the presence of a minor suppressive subpopulation of TANs cannot be excluded, our data suggest that in patients with early stage lung cancer, TANs do not significantly contribute to the inhibition of T cell responses [19]. Freshly isolated TANs from early stage lung cancer patients were not able to suppress IFN-γ production or proliferation of T cells that had been activated with anti-CD3/CD28 antibodies or allogeneic dendritic cells (DC). In fact, the TANs isolated from a vast majority of small-sized, early stage tumors were actually able to stimulate T-cell response to varying degrees [19]. Direct cell–cell contact was important for this neutrophil-mediated stimulation of T-cell proliferation. The important feature of this interaction is cross talk between TANs and T cells resulting in mutual cell activation (Fig. 2). During their interaction, T cells further up-regulated activation markers and produced more IFN-γ, whereas TANs showed increased longevity and expressed the co-stimulatory molecules CD86, CD54, OX40L, and 4-1BBL [19]. Typically, these co-stimulatory molecules are expressed on antigen-presenting cells, including mature dendritic cells, activated macrophages, and B cells [43]. Our data suggest that the CD86, CD54, 4-1BBL and OX40L co-stimulatory molecules can also be up-regulated on activated TANs as a result of their interaction with activated T cells. The ability of human neutrophils to up-regulate OX40L has also been reported in human sepsis [44]. The OX40L/OX40 and 4-1BBL/4-1BB pathways could have the potential to enhance anti-tumor immunity and break tumor-induced immune suppression and immunological tolerance. Indeed, the administration of soluble OX40L or gene transfer of OX40L into tumors has been shown to strongly enhance anti-tumor immune function in mice [45]. Furthermore, co-stimulation through 4-1BB/CD137 protects from activation-induced cell death and enhances the anti-tumor effector function of CD8$^+$ melanoma tumor-infiltrating lymphocytes [46]. Our data are in good concordance with the previous studies demonstrating the ability of granulocytes to provide accessory signals for T cell activation [16, 39]. For example, Radsak et al. have shown that PMNs activated with IFN-γ and GM-CSF are able to augment T-cell proliferation by providing co-stimulatory signals through MHC class II,

CD86, and CD54 co-stimulatory molecules. Our findings demonstrated that some lung tumors can secrete IFN-γ and GM-CSF, as well as trigger the expression of co-stimulatory molecules, including CD86 and CD54, on the surface of TANs. Furthermore, using an in vitro functional assay, we found that blocking CD86, CD54, OX40L or 4-1BBL with neutralizing antibodies significantly reduced the ability of TANs to stimulate T cells [19]. Thus, the early stage tumors have a potential to create a favorable environment in which TANs can bolster T-cell response.

Importantly, our study also showed that this T-cell stimulatory activity was related to the size of early stage tumors [19]. In contrast to the stimulatory interactions described above, we observed that when TANs were isolated from large early stage tumors, they attenuated T-cell stimulatory ability. It appears that the anti-tumoral potential of neutrophils diminishes during tumor progression, supporting the concept of an immunogenic ''switch'' from anti-tumor to pro-tumor phenotype [47, 48]. We speculate that, while small-sized early stage tumors in humans have already reached a considerable size and represent the final escape stage of the immunoediting process, the process of tumor evolution is still ongoing, because the profound immunosuppressive environment is not fully developed yet and anti-tumoral neutrophils still prevail in small-size tumors (early escape stage). The resistant tumor clones, which survived the immunoediting process, likely develop further immunosuppressive mechanisms that sustain tumor growth into even more advanced stages. At these advanced tumor stages, the suppressive environment appears to disable anti-tumoral N1 neutrophils and perhaps even convert them into the pro-tumoral N2 type (late escape stage). Thus, it is very possible that N2 pro-tumoral TANs represent the consequence of tumor progression, rather than the cause. This hypothesis has not been experimentally confirmed, but we are currently testing it by studying TANs from patients with advanced lung cancer (stages III and IV). However, obtaining these cells is logistically challenging, since these individuals do not routinely undergo tumor resection and are managed with chemotherapy and radiation therapy. In support of this concept, in studies using murine TANs with a very compressed time scale compared to human tumors, Mishalian et al. reported that TANs from small early tumors (Days 1–7 after implantation) were cytotoxic to tumor cells and produced higher levels of TNF-α, NO, and H_2O_2 compared with TANs in larger, established tumors [48]. It appears that TANs isolated from small-size, early stage lung cancers resemble the anti-tumor N1 TANs from small, early murine tumors, and as tumors become larger, the TANs become less stimulatory and lose this N1 phenotype.

TANs with composite characteristics of neutrophils and APCs

As mentioned above, in some lung cancer patients, we identified a specialized subpopulation of TANs that exhibited hybrid characteristics of canonical neutrophils and APCs (APC-like hybrid TANs) [20]. These APC-like hybrid TANs accumulated only in small, early stage tumors that produced low amounts of IFN-γ and GM-CSF. These APC-like hybrid neutrophils originated from immature $CD15^{hi}CD66b^{+}CD10^{-}CD16^{-/low/int}$ progenitors and were driven to differentiate into the hybrid phenotype by IFN-γ and GM-CSF present within the tumor microenvironment in a subset of patients with early stage lung cancers [20]. These soluble factors, at the very low concentrations found in tumor-conditioned media, synergistically exert their APC-promoting effect on immature neutrophils via the down-regulation of the transcription factor Ikaros [20]. Interestingly, the development of APC-like hybrid neutrophils was inhibited under hypoxic conditions [20]. This observation might explain their absence in large tumors. APC-like hybrid TANs acquire new functions compared to canonical TANs and are able to: (1) augment both antigen non-specific and tumor-specific T- cell responses, (2) directly stimulate antigen-specific autologous memory and effector T-cell responses to virus and tumor-derived antigens, respectively, and (3) uptake, degrade, and cross-present tumor antigens [20]. Interestingly, in our model antigen system, we found that antigen cross-presentation is triggered in hybrid neutrophils only when tumor antigen (in this case, NY-ESO-1 protein) was delivered as an IgG-immune complex, although this cross-presentation occurred at a relatively low level [20]. Therefore, our data demonstrate that APC-like hybrid neutrophils likely utilize their up-regulated FcγRI and FcγRII receptors for efficient antigen uptake and cross-presentation. It is also possible that these hybrid neutrophils may ''regurgitate'' processed peptide outside of the cell and thus facilitate the antigen uptake and processing by other professional APCs that are frequently co-localized with neutrophils in lung tumors [40]. We also found a small population of APC-like neutrophils in regional lymph nodes of cancer patients (consistent with their expression of the lymph node homing receptor, CCR7), suggesting they play a potential role in directly priming effector T-cell responses outside of the tumor. Our findings are in line with the previous studies demonstrating the ability of activated neutrophils to function as professional APCs in some inflammatory diseases [28–30]. In particular, it has been shown that activated mouse and human neutrophils are able to present viral and bacterial antigens to T cells and prime antigen-specific Th1 and Th17 cells [16, 29, 40, 49].

The strong T-cell stimulatory activity of hybrid neutrophils may contribute to the amplification of anti-tumor effector CD8 responses or the longevity of CD8 T-cell memory in early stage tumors. Thus, it is possible that these hybrid TANs are a primary contributor to the strong T-cell stimulatory effect of all TANs as observed in small-size, early stage tumors. Consistent with this idea, the absence of hybrid TANs and the establishment of an immunosuppressive tumor microenvironment may explain the inability of TANs to stimulate T cells in large tumors as described above.

Interestingly, we found that the differentiation of APC-like neutrophils into T-cell stimulatory cells strictly depends on the concentration of IFN-γ and GM-CSF in the tumor microenvironment. While low doses of IFN-γ and GM-CSF synergistically drove the differentiation of immature neutrophils into highly immunostimulatory hybrid neutrophils, high doses of IFN-γ resulted in the formation of hybrid neutrophils with high expression of PD-L1 and the ability to profoundly suppress T-cell responses [20]. This dual effect of APC-like hybrid neutrophils on T cells suggests a regulatory role for hybrid neutrophils in inflammation, in situations when stimulation of T cells should be followed by subsequent suppression to resolve the inflammatory process. On the other hand, in advanced stage tumors, where a more chronic inflammatory process exists, immature neutrophils might be directed to suppress anti-tumor T-cell response by converting immature granulocytes into G-MDSC, thus facilitating tumor growth.

We believe that early stage lung tumors exert diverse effects on the differentiation and function of TANs resulting in the formation of two subsets of TANs: canonical and hybrid [20]. Although recruited mature neutrophils acquire the phenotype of activated canonical TANs, immature neutrophils can change their differentiation program depending on the tumor microenvironment. We, therefore, envision a system in which, if tumors produce a sufficient amount of IFN-γ and GM-CSF, the immature neutrophils will differentiate into hybrid neutrophils. In the absence of these factors, or if other inhibitory conditions become dominant in the tumor microenvironment, then the immature neutrophils use a default pathway and become canonical TANs. Given that APC-like TAN frequency correlates inversely with tumor size, their role in modulating the host anti-tumor response in early tumor stages may be critical in limiting disease progression. It is tempting to speculate that by encouraging the development of APC-like TANs pharmacologically, these cells might provide an even more potent augmentation of the host anti-tumor T-cell response and reduce overall tumor burden.

Conclusion

In contrast to observations in murine model systems using rapidly growing, highly immunosuppresive tumor cells, human tumor development represents a process of slow and gradual evolution. Therefore, it is critical to understand the complex interaction of TANs with the tumor microenvironment at all stages of tumor evolution, since their relationship appears to change over time. Our findings characterize tumor-infiltrating neutrophils and their subsets in patients with early stage lung cancer for the first time. Areas of active investigation in our lab focus on the determination of the specific roles of canonical and APC-like hybrid TANs during early and advanced stages of tumor development in lung cancer patients. Deciphering the functional role of TANs in early versus advanced stages of lung cancer will add new knowledge to our understanding of TAN plasticity during tumor progression and may help us to develop different therapeutic strategies to regulate the function of TANs depending on tumor stage. Understanding how to direct and maintain human TANs towards anti-tumor effector cells will open new therapeutic options and aid in the future design of active immunotherapy to boost natural or vaccine induced anti-tumor immunity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer

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Infiltrating inflammatory cells are highly prevalent within the tumor microenvironment and mediate many processes associated with tumor progression; however, the contribution of specific populations remains unclear. For example, the nature and function of tumor-associated neutrophils (TANs) in the cancer microenvironment is largely unknown. The goal of this study was to provide a phenotypic and functional characterization of TANs in surgically resected lung cancer patients. We found that TANs constituted 5%–25% of cells isolated from the digested human lung tumors. Compared with blood neutrophils, TANs displayed an activated phenotype ($CD62L^{lo}CD54^{hi}$) with a distinct repertoire of chemokine receptors that included CCR5, CCR7, CXCR3, and CXCR4. TANs produced substantial quantities of the proinflammatory factors MCP-1, IL-8, MIP-1α, and IL-6, as well as the antiinflammatory IL-1R antagonist. Functionally, both TANs and neutrophils isolated from distant nonmalignant lung tissue were able to stimulate T cell proliferation and IFN-γ release. Cross-talk between TANs and activated T cells led to substantial upregulation of CD54, CD86, OX40L, and 4-1BBL costimulatory molecules on the neutrophil surface, which bolstered T cell proliferation in a positive-feedback loop. Together our results demonstrate that in the earliest stages of lung cancer, TANs are not immunosuppressive, but rather stimulate T cell responses.

Introduction

Murine and human studies suggest that tumor initiation and progression are commonly accompanied by "smoldering" inflammation (1). Tumor-infiltrating myeloid cells represent a significant proportion of the inflammatory cell population in the tumor microenvironment, and they influence nearly every step in tumor progression, including the suppression of adaptive immunity, the promotion of neoangiogenesis and lymphangiogenesis, the remodeling of the extracellular matrix, the promotion of invasion and metastasis, and lastly, the inhibition of vaccine-induced antitumor T cell responses (2). Among the different types of myeloid cells, tumor-associated macrophages (TAMs) have been the best characterized and are generally considered protumoral in murine tumor models (3, 4). The role of tumor-associated neutrophils (TANs) in cancer progression remains unclear and has been investigated only recently in murine models. Characterization of human TANs is even less well developed.

In murine studies, TANs appear to have dichotomous protumor and antitumor effects (5–7). Similar to the classic (M1) and alternative (M2) activation pathways proposed for TAMs, the paradigm of antitumor "N1 neutrophils" versus protumoral "N2 neutrophils" has been proposed in murine models (8). Whether these paradigms translate to human tumor biology remains unanswered. Critical species-specific differences in both innate and adaptive immunity make assumptions of equivalence unwise (9), especially given recent studies that have shown that certain rodent models poorly replicate inflammatory diseases in humans (10). In humans, correlative studies using immunohistochemistry have shown that TAN infiltrates are associated with a poor prognosis for patients with head and neck cancer (11), renal cell carcinoma (12), melanoma (13), hepatocellular cancer (14), and colon cancer (15). In contrast, high tumor neutrophil counts have been associated with a favorable outcome for patients with gastric cancer (16). The results in lung cancer have been divergent (17, 18). To our knowledge, there have been no reports regarding the functional role of TANs in the progression of human cancers. Thus, one goal of this work was to determine the phenotype and function of TANs in early-stage lung cancer using fresh surgically obtained tumor.

A major challenge in TAN biology is deciphering the complex interaction of activated neutrophils with T cells in the tumor microenvironment. Understanding the role of TANs in regulating T cell responses in cancer patients is particularly important because cytotoxic T lymphocytes are the chief effector cells mediating antigen-driven antitumor immunity. There is evidence that activated neutrophils can interact with T cells in dichotomous ways. Several studies have shown that neutrophils can present antigens

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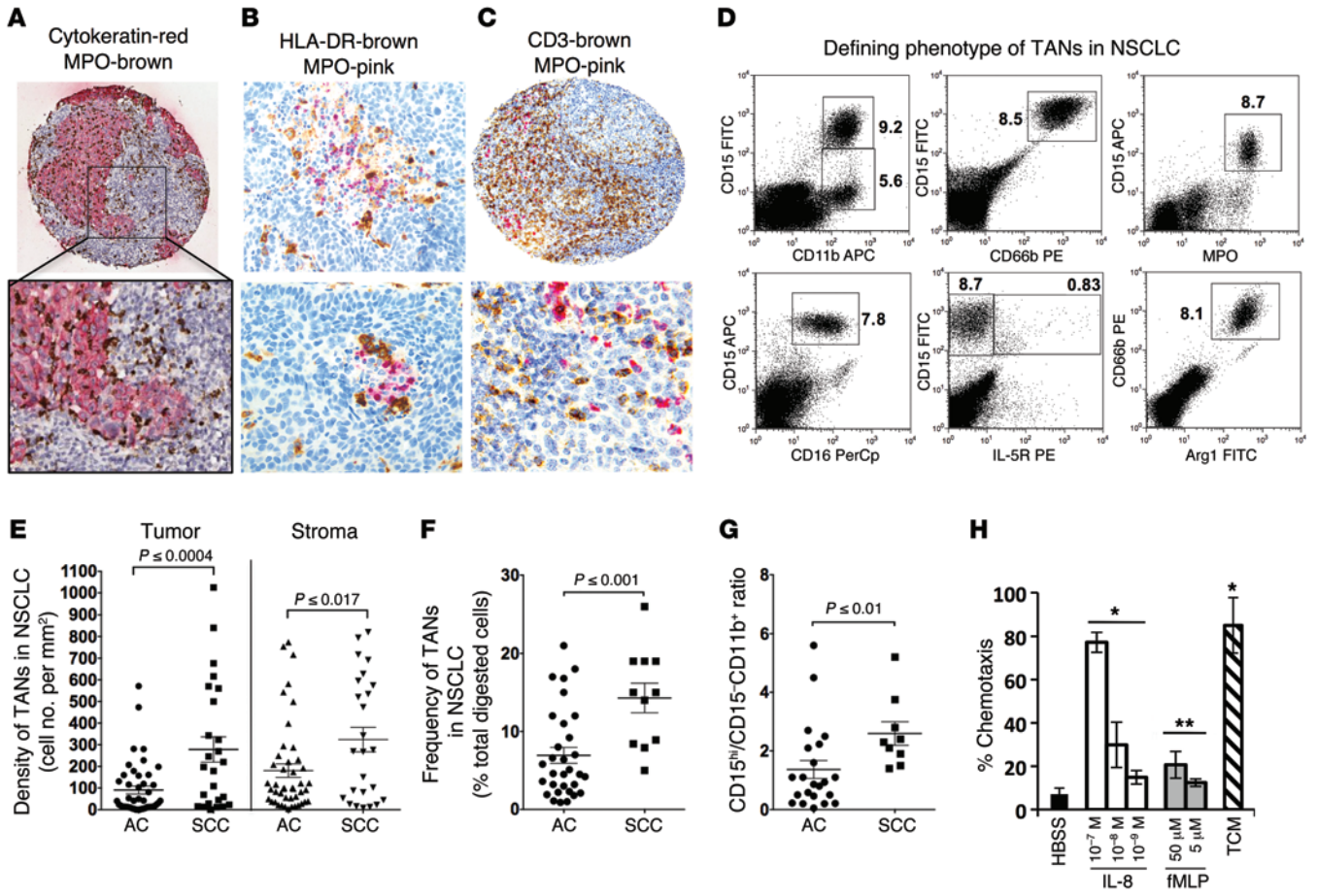

Figure 1. Neutrophils infiltrate NSCLC tissue. (**A**–**C**) Lung cancer tissue sections were stained using 2-color immunohistochemistry for MPO, HLA-DR, and CD3 to visualize neutrophils, APCs, and T lymphocytes, respectively. Representative images are shown. Original magnification, ×10 (**A** and **C**, top), ×20 (**B**, top), ×40 (bottom). (**D**) Representative dot plots of total tumor cells that define the phenotype of TANs in NSCLC. TANs were defined as $CD15^{hi}CD66b^{+}CD11b^{+}CD16^{int}Arg1^{+}MPO^{+}IL\text{-}5Ra^{-}$. Results represent 1 of 20 experiments. Numbers represent the percentage of TANs. (**E**) Comparative immunohistochemical analysis of TAN density (cells/mm²) in AC (n = 45) and SCC (n = 25) performed by counting of MPO^{+} cells in the tumor stroma and the tumor islets. Statistical analyses were performed with Student's t test for unpaired data. (**F**) The frequency of TANs in AC (n = 31) and SCC (n = 11) determined by flow cytometry as the percentage of $CD11b^{+}CD15^{hi}CD66b^{+}$ cells among all cells in tumor. Cumulative results from 42 independent experiments are shown in the scatter plot. Student's t test for unpaired data. (**G**) The ratio of TANs to other $CD15^{-}CD11b^{+}$ cells in AC (n = 22) and SCC (n = 9). Mann-Whitney test for unpaired data. For all scatter plots, error bars represent mean ± SEM. (**H**) PBNs were analyzed for migration in the Neuro Probe ChemoTx system. Each experiment was run in triplicate and repeated at least 3 times. Results of 1 representative experiment are shown. Error bars represent mean ± SEM. Statistical analysis was performed with Kruskal-Wallis and Dunn's multiple comparison tests (*$P \le 0.05$, **$P \le 0.01$). fMLP, N-formyl-methionyl-leucyl-phenylalanine.

and provide accessory signals for T cell activation (19–22). Other studies have suggested that peripheral blood neutrophils (PBNs) can suppress antigen-nonspecific T cell proliferation through the release of arginase-1 and the production of ROS (23–25). To date, the suppressive function of granulocytic cells in cancer patients has generally been attributed to a circulating low-density granulocytic myeloid-derived suppressor cell (G-MDSC) population (26–28). However, there is some uncertainty about whether G-MDSCs exist in humans and whether they are simply a sequela of disease progression. Thus, given the unclear role of neutrophils in the regulation of T cell responses, a second major goal of this study was to determine the effects of TANs on T cell activation.

Results

Intratumoral neutrophils constitute a significant portion of infiltrating cells in lung cancers. To identify and localize TANs, sections from tumor microarrays containing 45 adenocarcinomas (ACs) and 25 squamous cell carcinomas (SCCs) were double-stained for cytokeratin to identify tumor cells (red) and myeloperoxidase (MPO) to identify TANs (brown) (Figure 1A). The median numbers of MPO^{+} cells present in the tumor islets and stroma in AC (40 cells/mm² and 97 cells/mm², respectively) were significantly less ($P < 0.02$) than those seen in SCC (197 cells/mm² and 269 cells/mm², respectively) (Figure 1E).

Double staining of tumor sections with MPO and HLA-DR or CD3 revealed that neutrophils often colocalized with APCs (Figure 1B) and T cells (Figure 1C) throughout the lung tumor microenvironment. In some cases, TANs were more associated with microabscesses or coagulative necrosis (Figure 1B, top).

To perform a more detailed evaluation of TANs by multicolor flow cytometry, the generation of high-quality single-cell suspensions is required. We tested several commercially available

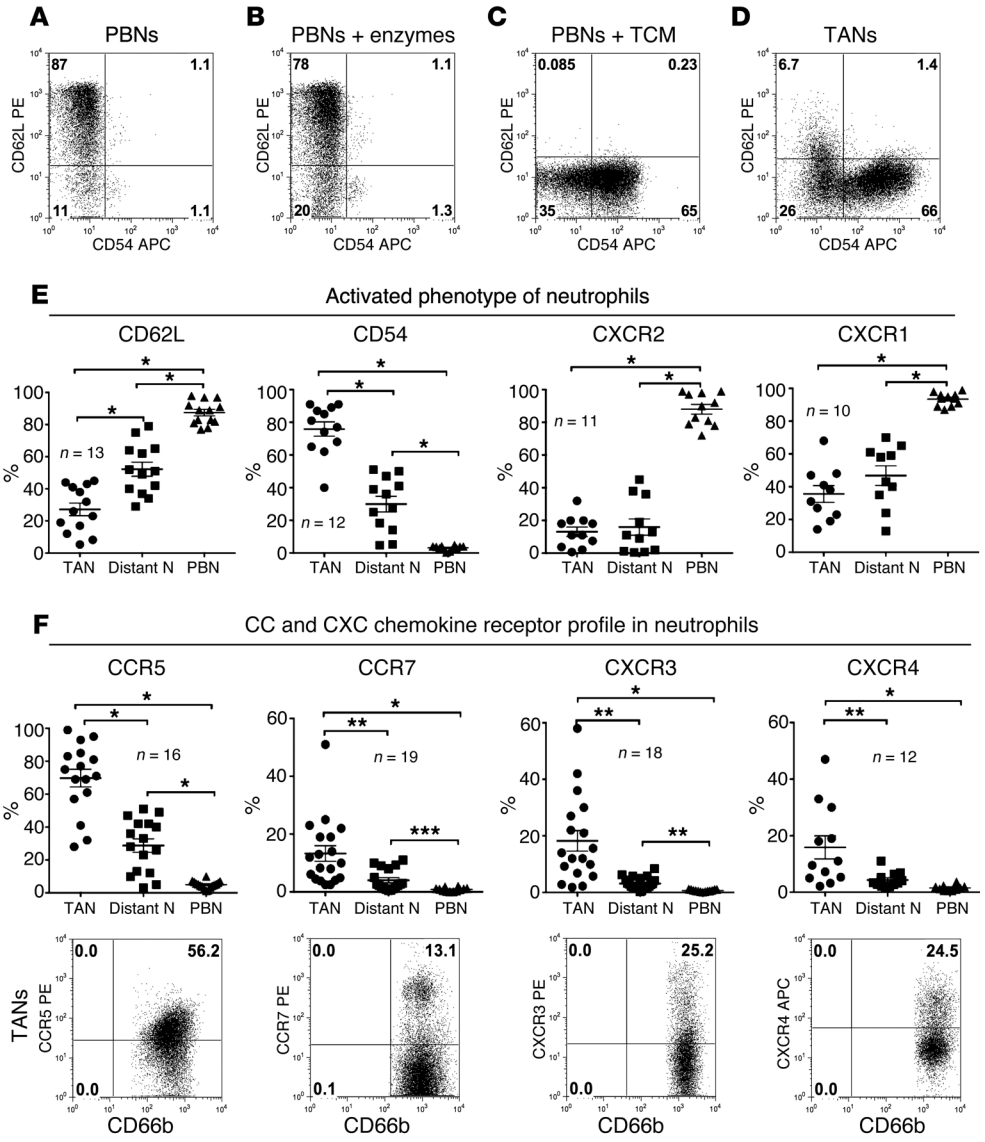

Figure 2. TANs acquire an activated phenotype and novel repertoire of chemokine receptors. (**A**) Expression of the activation markers CD62L and CD54 on $CD15^{hi}CD66b^{+}$ PBNs. PBNs were isolated from lung cancer patients using anti-CD15 beads. Results represent 1 of 5 experiments. (**B**) Digestion protocol did not elicit premature activation of resting PBNs. Results represent 1 of 5 experiments. (**C**) PBNs acquire an activated $CD62L^{lo}CD54^{+}$ phenotype after treatment with TCM in plates with ultralow attachment surface. Each experiment was repeated at least 5 times. (**D**) A single-cell suspension was obtained from freshly harvested tumor tissues. TANs were gated on $CD11b^{+}CD15^{hi}CD66b^{+}$ cells and further analyzed for the expression of activation markers. TANs displayed an activated $CD62L^{lo}CD54^{+}$ phenotype. Results represent 1 of 12 experiments. (**E**) Expression of the activation markers on gated $CD11b^{+}CD15^{hi}$ TANs, distant lung neutrophils (Distant N), and PBNs. (**F**) Expression of CCR5, CCR7, CXCR3, and CXCR4 was analyzed on gated $CD11b^{+}CD15^{hi}CD66b^{+}$ TANs, distant lung neutrophils, and PBNs of cancer patients. Bottom: Representative dot plots. Numbers represent the percentage of cells in each quadrant. Top: Summary of all patient data. Error bars represent mean ± SEM. Statistical analyses were performed with repeated-measures 1-way ANOVA with Tukey's multiple comparison test for CD62L, CD54, CXCR2, CXCR1, and CCR5, and Kruskal-Wallis and Dunn's multiple comparison tests for CCR7, CXCR3, and CXCR4 ($*P \le 0.001$, $**P \le 0.01$, $***P \le 0.05$).

and individually customized enzymatic cocktails to optimize cell yield, cellular viability, myeloid cell population recovery, preservation of myeloid cell surface marker expression, and induction of neutrophil activation (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI77053DS1). We selected a combination cocktail (described in Methods) that was composed of several enzymes used at low concentrations and led to a high yield of viable single cells (Supplemental Figure 1, A and B) with minimal enzyme-induced ex vivo effects on neutrophil activation (Figure 2, A and B) and cleavage of myeloid and lymphoid cell markers (Supplemental Figure 1, C–G). Once optimized, we studied tumors from 86 non–small-cell lung carcinoma patients with stage I–II SCC and AC histology. The detailed patient characteristics are shown in Supplemental Table 1.

Single-cell suspensions from these fresh human lung tumors were stained for neutrophil markers (CD15, CD66b, MPO, and arginase-1 [Arg1]), myeloid lineage markers (CD11b, CD16, and CD33), and the eosinophil marker IL-5Rα, by flow cytometry. TANs were defined as $CD15^{hi}CD66b^{+}CD11b^{+}MPO^{+}Arg1^{+}CD16^{int}$ cells (Figure 1D). Importantly, the $CD15^{hi}CD66b^{+}CD11b^{+}$ granulocytes had negligible expression of the eosinophil-associated surface marker IL-5Rα (Figure 1D). In multicolor tracings, we defined TANs as either $CD15^{hi}CD11b^{+}$ or $CD66b^{+}CD11b^{+}$, since there was a 100% concordance between CD15 and CD66b (Figure 1D).

The $CD15^{hi}CD11b^{+}$ TANs were found to be present in varying frequencies, ranging from 2% to 25% of live cells in the tumor microenvironment in all of the NSCLC studied (Figure 1F). In agreement with the immunohistochemical data (Figure 1E), the frequency of TANs and the $CD15^{hi}CD11b^{+}$ to $CD15^{-}CD11b^{+}$ ratio were significantly higher ($P = 0.001$) in patients with SCC (~15% of live cells; ratio of 2.6) compared with patients with an AC (~7% of live cells; ratio of 1.4) (Figure 1, F and G). This indicates that $CD15^{hi}CD11b^{+}$ TANs constituted the major proportion of $CD11b^{+}$ tumor-infiltrating myeloid cells in patients with SCC. It is noteworthy that the proportion of TANs in tumor tissue of both histological types did not correlate with tumor size (Supplemental Table 2 and Supplemental Figure 5, A–C). Importantly, the density of intratumoral neutrophils was not associ-

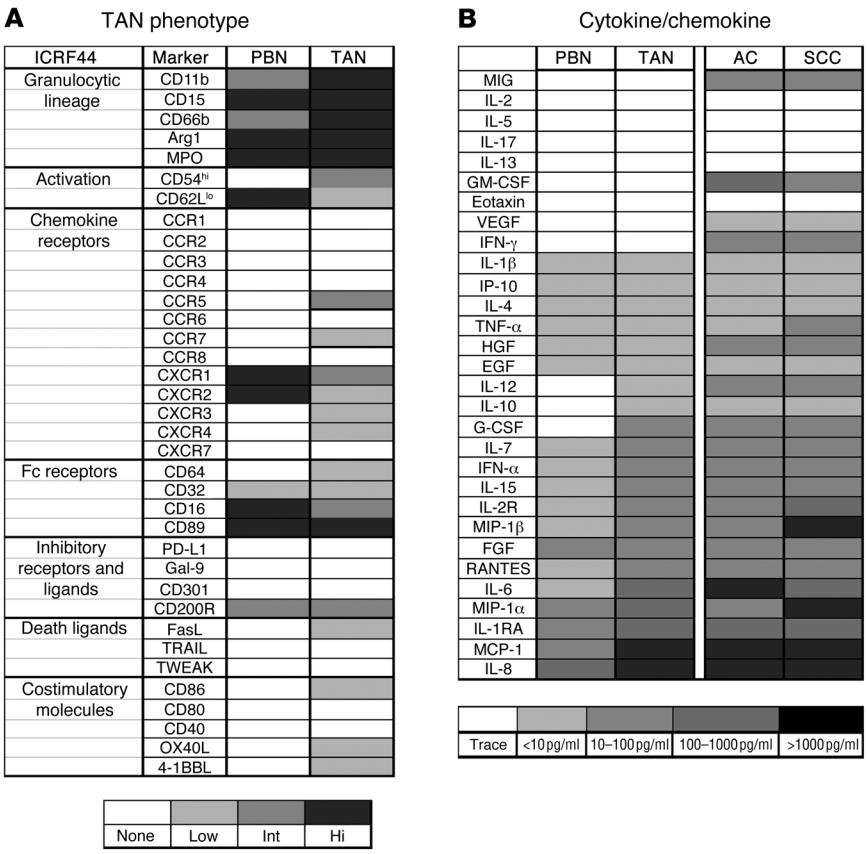

Figure 3. Characterization of TANs. (**A**) Heat map comparing the phenotypes of TANs and PBNs. A single-cell suspension was obtained from freshly harvested tumor tissues, and expression of the indicated markers was assessed using flow cytometry. TANs were gated on $CD11b^{+}CD15^{hi}$ cells and further analyzed for the expression of indicated markers. PBNs were treated similarly to TANs. Expression of each marker was analyzed in 10–18 patients. The intensity key for the heat map is shown below. (**B**) The cytokine/chemokine production by TANs, PBNs, and total tumor dissociates of AC and SCC. TANs and PBNs were isolated from tumor tissues and peripheral blood of lung cancer patients (n = 5) using magnetic beads. Purified neutrophils and unseparated cells from digested tumor were cultured for 24 hours in the cell culture medium, and cell-free supernatants were collected and frozen. The indicated factors were detected using the Cytokine Human 30-Plex assay. The presence of each secreted factor was heat-mapped on the basis of the concentration in tested supernatants, as indicated below.

ated with smoking use in the patients (Supplemental Table 2 and Supplemental Figure 5D).

To determine whether the tumor microenvironment stimulates trafficking of neutrophils, resting PBNs were assayed for transwell migration in the presence of tumor-conditioned medium (TCM) collected from digested tumors. In this assay, we observed that TCM induced a strong chemotactic response in blood neutrophils (Figure 1H). In fact, TCM was as efficient as high concentrations of IL-8, a known neutrophil chemotactic factor, in attracting $CD15^{+}$ granulocytes (Figure 1H).

Neutrophils are activated in the tumor microenvironment in patients with NSCLC. Since changes in cell adhesion molecules (CD62L, CD54) and CXC chemokine receptors (CXCR1, CXCR2) have been reported to correlate with leukocyte activation, augmented chemotaxis, and transendothelial migration (29, 30), these markers were measured on circulating and tumor-associated $CD11b^{+}CD15^{hi}$ neutrophils. There was no significant difference between lung cancer patients and healthy donors in the expression of these markers on PBNs; these neutrophils shared the phenotype of resting naive cells, i.e., $CD62L^{hi}CD54^{-}CXCR1^{hi}CXCR2^{hi}$ (Figure 2, A and E).

Previously, others have described a distinct subset of activated $CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$ low-density granulocytes in the PBMC fraction of patients with advanced stages of NSCLC (31). This population has been referred to as granulocytic myeloid-derived suppressor cells (G-MDSCs), because of their ability to suppress T cell proliferation (32). We analyzed PBMCs from 20 healthy donors and 20 lung cancer patients at early stages of disease for the presence of G-MDSCs and found that their frequency in cancer patients was low and not significantly different from the frequency in healthy donors (0.9% ± 0.17% and 0.7% ± 0.18%, respectively).

Next, we evaluated the expression of activation markers on $CD11b^{+}CD15^{hi}$ TANs and neutrophils isolated from lung tissue adjacent to the lung cancer (Distant N; Figure 2E). Notably, the digestion protocol did not elicit premature activation of resting PBNs (Figure 2B). In contrast to PBNs, $CD11b^{+}CD15^{hi}$ TANs markedly upregulated CD54 and downregulated CD62L, CXCR1, and CXCR2 (Figure 2, D and E), acquiring the phenotype of highly activated cells ($CD62L^{lo}CD54^{+}CXCR1^{lo}CXCR2^{lo}$). However, when resting PBNs were isolated from healthy donors and cultured in the presence of primary TCM, they rapidly acquired an activated $CD62L^{lo}CD54^{hi}$ phenotype, suggesting that tumor-derived factors are sufficient to trigger these changes (Figure 2C). Similarly to TANs, neutrophils from distant noninvolved lung downregulated the expression of CXCR1 and CXCR2 when compared with PBNs. However, the analysis of the CD54 and CD62L expression demonstrated that distant neutrophils were less activated compared with TANs (Figure 2E). There were no differences in the expression of activation markers on TANs between patients with AC and SCC (Supplemental Table 2).

Phenotypic changes in tumor-infiltrating neutrophils. There is growing evidence that the inflammatory microenvironment may induce a novel chemokine receptor repertoire on infiltrating neutrophils that increases their functional responsiveness to surrounding chemokines (33). Thus, we determined whether neutrophils gained a new chemokine receptor expression profile in lung tumors.

Peripheral blood and tumor-associated $CD11b^{+}CD15^{hi}CD66b^{+}$ neutrophils from patients with lung cancer were assayed for a wide

array of CC (CCR1–CCR7) and CXC (CXCR1–CXCR7) chemokine receptors. Subpopulations of TANs expressed CCR7, CXCR3, and CXCR4, whereas these chemokine receptors were absent on PBNs (Figure 2F and summarized in Figure 3A). Virtually all TANs expressed high levels of CCR5, which was absent on PBNs (Figure 2F). Interestingly, the proportion of CCR5-positive TANs was significantly higher in patients with AC compared with SCC ($P = 0.04$) (Supplemental Table 2). In addition, CXCR1 and CXCR2 were dramatically downregulated on TANs and distant neutrophils (Figure 2E). The expression of CCR5, CCR7, and CXCR3 on the distant neutrophils was significantly higher in comparison with PBNs ($P < 0.01$) and significantly lower in comparison with TANs ($P < 0.01$). These differences in chemokine receptor expression were consistent, regardless of whether they were measured by cell surface expression (MFI) or as a percentage of chemokine receptor–positive neutrophils. Importantly, there were no differences in the expression of CC and CXC receptors on PBNs between healthy donors and patients with stages I–II NSCLC (data not shown).

The activation of neutrophils has also been suggested to lead to the upregulation of inhibitory receptors and ligands that negatively regulate T cell responses (34–37). However, the expression of PD-L1, galectin-9, CD200R, and CD301 was not increased on TANs in 10 patients with early stages of AC or SCC (Figure 3A). In addition, we found that TANs expressed low levels of FasL and the FcRI receptor CD64 (albeit higher than PBNs), whereas the expression of the FcγRIII receptor CD16 was downregulated compared with that seen on PBNs (Figure 3A).

Engagement of CD15 or CD66b molecules in the isolation of TANs. Given studies that show minimal effects of positive selection of granulocytes by anti-CD15 Ab–conjugated magnetic microbeads on ROS production or phagocytosis (38), we used a combination of our tumor digestion protocol and anti-CD15 microbeads to isolate granulocytes from digested tumors and peripheral blood for functional studies. We first treated peripheral blood of healthy donors with the enzymatic cocktail, and then isolated neutrophils using anti-CD15 microbeads. Analysis of the expression of the activation markers CD62L and CD54 on PBNs indicated that these cells were not prematurely activated or adversely affected by this process (Figure 2, A and B). Using this approach, we found that isolation of TANs with the anti-CD15 microbeads yielded high neutrophil purity, as defined by the $CD15^{hi}CD66b^{+}CD11b^{+}MPO^{+}Arg1^{+}$ phenotype (Supplemental Figure 3A). We verified the cellular purity of TANs in every patient by flow cytometry and cytomorphology. TANs isolated with purity less than 90% were discarded. Over 95% of the sorted cells expressed the neutrophilic markers CD11b, MPO, CD66b, and Arg1. Cytospins were prepared from isolated TANs, and pathological review confirmed that the cytomorphology of these cells was consistent with granulocytes (Supplemental Figure 3D). Importantly, annexin V and propidium iodide (PI) staining of isolated $CD15^{+}$ PBNs showed that only 9% ± 3% of the cells were in apoptosis, indicating that significant cell death was not occurring during the isolation procedures (Figure 4, A and B). $CD15^{hi}CD66b^{+}CD11b^{+}$ TANs isolated from the majority of patients with lung cancer were MPO and Arg1 positive (Supplemental Figure 3, B and C). However, in some patients we found that the fraction of TANs had reduced intracellular MPO and Arg1, suggesting that these enzymes had already been released in the tumor tissue before isolation. Isolation of TANs and PBNs using positive selection with engagement of CD66b demonstrated similar results (data not shown).

Cytokine/chemokine profile of TANs and total NSCLC cells. To describe the range of inflammatory factors secreted by neutrophils in lung cancer patients, 24-hour cell culture supernatants from purified TANs and PBNs were analyzed by a multiplexed fluorescent bead–based immunoassay. In addition, we analyzed these factors in the supernatants collected from total cells of digested AC or SCC. The heat map in Figure 3B shows that many chemokines and cytokines were upregulated in the TAN group. TANs isolated from patients with AC or SCC, compared with PBNs from the corresponding patients, had significantly increased ($P < 0.05$) production of the proinflammatory factors MCP-1, IL-8, MIP-1α, and IL-6 (Figure 3B). Importantly, TANs were able to simultaneously secrete considerably more of the antiinflammatory IL-1R antagonist compared with PBNs.

Interestingly, the analysis of cytokines secreted by digested tumors harvested from 5 patients with AC and 5 patients with SCC revealed a relative preponderance of the Th1 proinflammatory cytokines IFN-γ (100 ± 58 pg/ml), IL-12 (18 ± 4 pg/ml), and TNF-α (81 ± 27 pg/ml), compared with the Th2 cytokines IL-4 (2.6 ± 1.8 pg/ml), IL-5 (not detectable), IL-10 (9.8 ± 3 pg/ml), and IL-13 (not detectable) (Figure 3B). The concentration of the proangiogenic cytokine VEGF was extremely low (8 ± 2.7 pg/ml) in the supernatants from digested AC and SCC. Several prominent NSCLC-derived cytokines, such as IL-8, MCP-1, IL-1RA, GM-CSF, and MIG, were present in high quantities in the cell culture supernatants collected from total cells of AC and SCC. However, there were some significant differences ($P < 0.05$) in the cytokine production between the tumor types. Compared with AC, SCC had significantly increased secretion of MIP-1α, MIP-1β, TNF-α, and IL-2R. In contrast, AC produced significantly ($P < 0.05$) more IL-6 and GM-CSF. MIP-1α has been shown to promote neutrophil chemotaxis (39); therefore its increased production in SCC may explain the high number of TANs relative to AC (Figure 3B).

TANs exhibit high phagocytic activity, and the ability to generate ROS in vitro. To assess functionality, PBNs and TANs were isolated from the same cancer patient and evaluated for their ability to survive in culture, phagocytose bacteria, and produce ROS in vitro.

There was no significant difference in the number of apoptotic cells in freshly isolated PBNs and TANs (5% ± 2.8% and 9% ± 3% of annexin $V^{+}PI^{-}$ and annexin $V^{+}PI^{+}$ cells, $P = 0.4$, respectively) (Figure 4A, top dot plots, and Figure 4B). After 20 hours in culture, there was no survival advantage for TANs compared with PBNs (Figure 4A, center dot plots, and Figure 4B). However, when either freshly isolated PBNs or TANs were cultured in the presence of TCM, these cells had substantially increased survival time compared with PBNs and TANs that were cultured in standard medium (Figure 4, A and B). About 40% of PBNs and TANs cultured in the presence of TCM were still viable at 48 hours (Figure 4B). These data suggest that tumor-derived factors prolong neutrophil survival in the tumor microenvironment.

Next, we examined the phagocytic activity of TANs and resting PBNs by measuring the uptake of red fluorescent pHrodo *E. coli* bioparticles. The results showed that TANs phagocytosed the bioparticles as efficiently as PBNs (Figure 4D). There was no

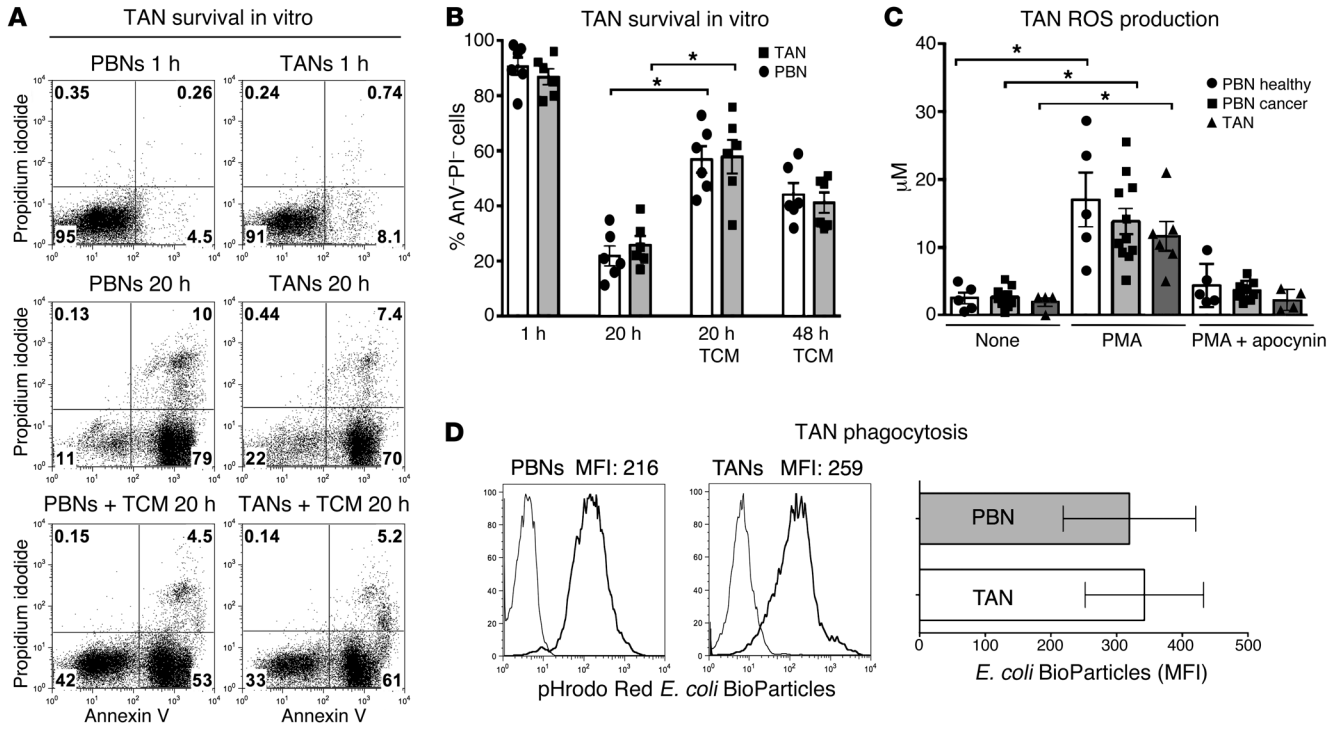

Figure 4. Characterization of neutrophils isolated from tumor tissues and peripheral blood of patients with NSCLC. (**A** and **B**) Neutrophil survival in vitro. TANs and PBNs were isolated from lung cancer patients with magnetic beads by positive selection. Cells were cultured in cell culture medium with or without TCM for 20 and 48 hours. TANs and PBNs were then assessed for apoptosis by FACS analysis of annexin V/PI staining at 1, 20, and 48 hours. Dot plots represent 1 of 6 similar experiments. Numbers represent the percentage of cells in each quadrant. Summary results from 6 lung cancer patients are also shown (*$P \leq 0.01$, Wilcoxon matched-pairs rank test). (**C**) Production of H_2O_2 in TANs and PBNs isolated from lung cancer patients and healthy donors was measured using Amplex Red with horseradish peroxidase. Error bars represent mean ± SEM from 5 independent experiments (*$P \leq 0.001$, Wilcoxon matched-pairs rank test). (**D**) Phagocytic capacity of TANs. TANs and PBNs were isolated and incubated with pHrodo Red *E. coli* BioParticles for 45 minutes to allow phagocytosis (internalized particles become fluorescent [red]). Histograms from 1 representative experiment are shown. Phagocytosis performed at 4°C and 37°C is shown by thin and thick lines, respectively; MFI values are as indicated in histograms. Summary results from 6 lung cancer patients are also shown (Wilcoxon matched-pairs rank test).

difference in the phagocytic activity between PBNs from cancer patients and healthy donors (data not shown).

We also quantified the spontaneous and PMA-stimulated ROS production by circulating and tumor-associated neutrophils using the Amplex Red assay. Despite the fact that TANs were activated, spontaneous ROS production was minimal and no different from that of resting PBNs from lung cancer patients or healthy donors (Figure 4C). However, the stimulation of PBNs and TANs with PMA resulted in a dramatic increase in H_2O_2 production, suggesting that TANs were not dysfunctional and could be stimulated further (Figure 4C). Mechanistically, ROS production in the neutrophils was mediated by the NADPH oxidase complex (NOX), since coculture of the TANs with the NOX inhibitor apocynin substantially decreased PMA-induced ROS generation (Figure 4C).

Together, our data on isolated TANs show that when appropriately triggered, they can perform major functions such as phagocytosis and ROS production, suggesting that they are not "exhausted" or hypofunctional.

Neutrophils from malignant and nonmalignant lung tissue stimulate antigen-nonspecific T cell proliferation. Multiple reports suggest that MDSCs in human and murine tumors are partially composed of granulocytic cells, and that these populations tend to negatively modulate effector T cell functions (27, 40). Accordingly, we measured the effect of TANs on lymphocyte proliferation in a CFSE-based T cell suppression assay. First, in order to validate this assay in our hands, we isolated Tregs (effectors) from the peripheral blood of lung cancer patients and cocultured them with CFSE-labeled healthy donor PBMCs (responders) that had been stimulated with plate-bound anti-CD3 Abs. As expected, after 4 days, we found that the proliferation of both $CD4^+$ and $CD8^+$ T cell populations exposed to Tregs was markedly suppressed compared with that of control cells (Supplemental Figure 2A).

Next, we performed similar experiments in which PBNs or TANs (effectors) were isolated from cancer patients and cocultured with healthy donor PBMCs (responders) that had been stimulated with plate-bound anti-CD3 Abs. We found that the proliferation of stimulated T cells after 4 days was not altered by exposure to PBNs (Figure 5A). Surprisingly, when the stimulated PBMCs were cocultured with TANs, the proliferation of $CD4^+$ and $CD8^+$ cells was markedly augmented. Specifically, whereas approximately 50% of control T cells were $CFSE^{lo}$ and underwent 1–6 rounds of cell division, the dividing T cell fraction significantly increased (to as much as 95%) in the presence of TANs (Figure 5A).

Isolation of TANs and PBNs using positive selection with engagement of the CD66b molecule demonstrated similar results (Supplemental Figure 2C). To confirm that our results were not

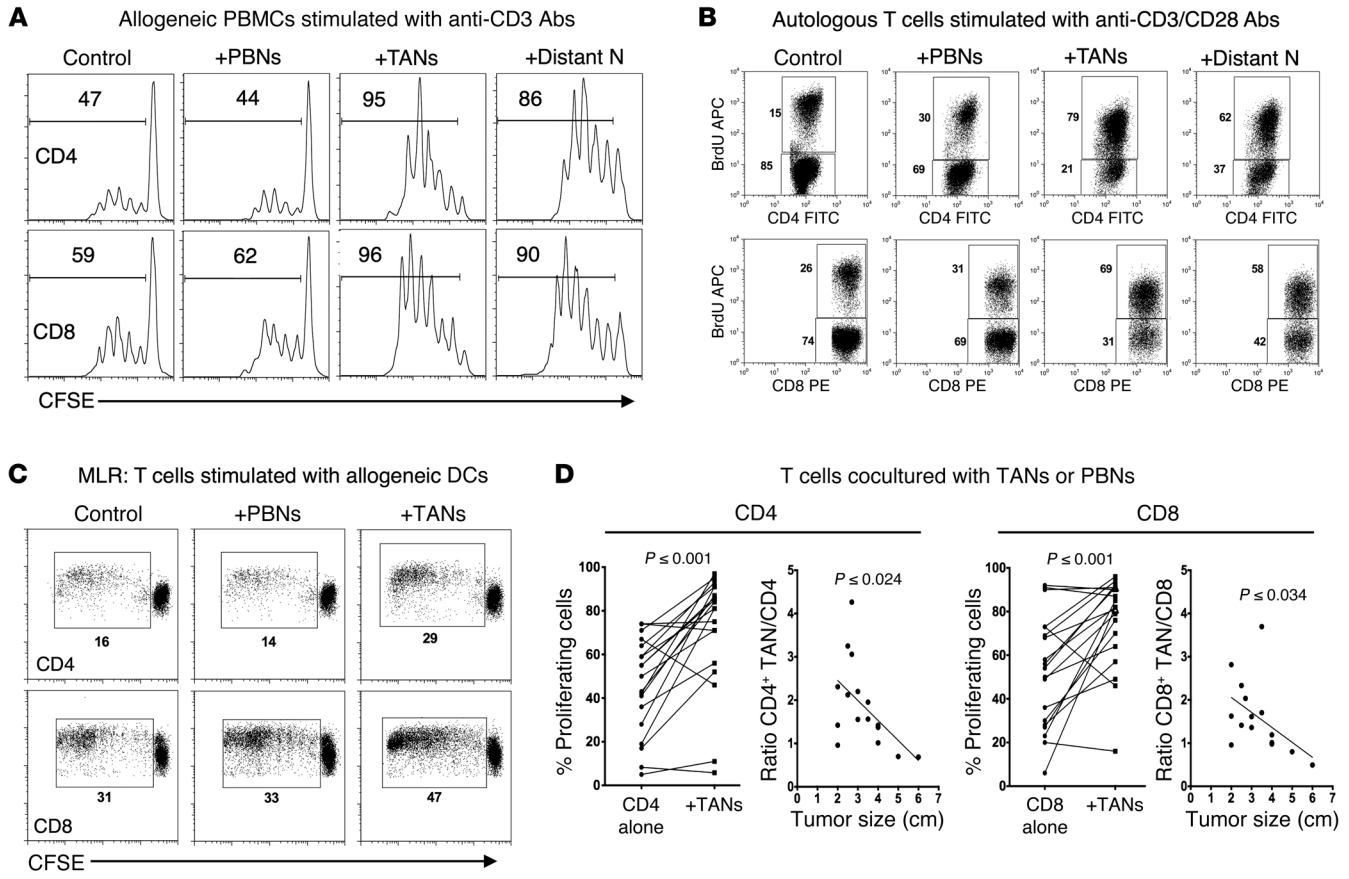

Figure 5. T cell proliferation in the presence of TANs or PBNs. (**A**) CFSE-labeled PBMCs isolated from a healthy donor were stimulated with plate-bound anti-CD3 Abs and mixed with TANs, neutrophils from distant lung tissue, or PBNs isolated from cancer patients at a 1:1 ratio for 4 days. Representative results from 1 of 12 experiments are shown. Numbers on histograms represent the percentage of proliferating T cells. (**B**) Autologous T cells were purified from PBMCs, stimulated with plate-bound anti-CD3 Abs and anti-CD28 Abs, and cultured alone or with PBNs, distant neutrophils, or TANs. 72 hours later, proliferation of T cells was assessed by incorporation of BrdU into DNA. Representative results from 1 of 5 experiments are shown. (**C**) Mixed lymphocyte reaction (MLR). CFSE-labeled T cells isolated from PBMCs of healthy donors were stimulated with allogeneic DCs in the absence or presence of PBNs or TANs for 5 days. Representative results from 1 of 5 experiments are shown. Numbers on dot plots represent the percentage of proliferating T cells. (**D**) Change in percentage of proliferating $CFSE^{lo}$ $CD4^+$ and $CD8^+$ cells cultured alone versus cells cultured in the presence of TANs (Student's *t* test, paired parametric test). Autologous PBMCs were stimulated with plate-bound anti-CD3 Abs and mixed with TANs at a 1:1 ratio for 4 days. Scatter plots show the correlation between tumor size and stimulatory activity of TANs defined as the ratio $CFSE^{lo}$ (T cells +TANs)/$CFSE^{lo}$ (T cells) (nonparametric Spearman correlation). Cumulative results from 16 independent experiments are presented.

due to contamination of our TANs by other tumor-infiltrating cells that may have escaped magnetic bead separation, we repeated the experiments using purified $CD45^+CD11b^+CD15^{hi}CD66b^+$ TANs collected by FACS. In triplicate experiments, the TANs sorted by flow cytometry again showed remarkable stimulatory effects on T cell proliferation (Supplemental Figure 2C).

To ensure that this stimulatory effect was not dependent on the allogenicity between healthy donor responders and patient TANs, or some artifact of the CFSE system, we repeated the T cell proliferation assay using T cells, PBNs, and TANs all isolated from the same patient. A BrdU incorporation assay demonstrated that the majority of T cells (79% of $CD4^+$ and 69% of $CD8^+$ cells) cocultured with TANs were in S phase of the cell cycle by 72 hours after stimulation, compared with only about 15%–30% of control T cells or T cells cocultured with PBNs (Figure 5B). This short-term assay revealed that activated T cells begin to actively synthesize DNA in the presence of TANs by 48 hours after stimulation compared with control T cells or T cells cocultured with PBNs (Supplemental Figure 2E). Interestingly, in this experiment, we found that PBNs and TANs partially liberated Arg1 in the presence of activated T cells (Supplemental Figure 3E). However, the presence of arginase did not seem to affect the rate of T cell proliferation. These data support other studies showing that the arginase, which is liberated following spontaneous polymorphonuclear neutrophil death, is not sufficient for T cell suppression (41).

Neutrophils isolated from distant nonmalignant lung tissue were also able to stimulate allogeneic and autologous T cell proliferation (Figure 5, A and B). However, there was no significant difference in stimulatory activity of distant neutrophils when compared with TANs (Supplemental Figure 2D). This suggests that stimulatory capacity of distant neutrophils is a lung tissue–specific characteristic that might be driven by adjacent early-stage lung tumor.

In order to quantify the extent to which TANs are able to increase T cell proliferation, we mixed TANs with autologous

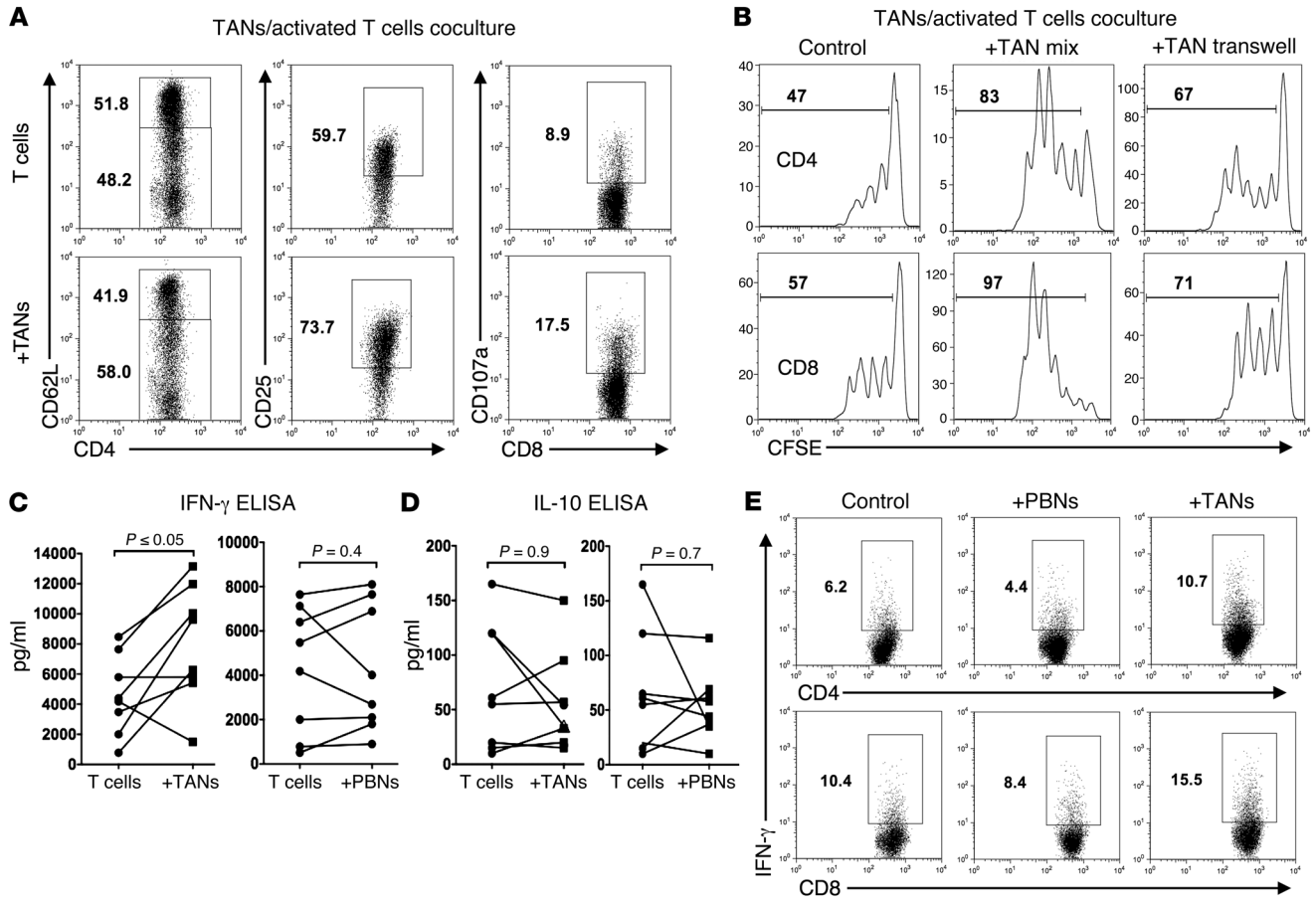

Figure 6. Effect of TANs on T cell activation, cytokine production, and proliferation. In all experiments, T cells were stimulated with plate-bound anti–CD3/CD28 Abs and incubated with TANs at a 1:1 ratio. (**A**) Expression of the CD62L, CD25, and CD107a markers on activated autologous T cells cultured with TANs for 20 hours. Representative dot plots from 1 of 3 experiments are shown. Numbers represent the percentage of $CD4^+$ and $CD8^+$ cells. (**B**) Flow cytometric analysis of autologous T cell proliferation in the presence of TANs using a transwell system. Activated CFSE-labeled T cells were mixed with TANs at a 1:1 ratio (TAN mix). To separate T cells and TANs, activated T cells were cultured in the bottom chamber and TANs were placed in the top chamber of the 24-well flat-bottom transwell culture plate (TAN transwell). Representative results from 1 of 3 experiments are shown. Numbers on histograms represent the percentage of proliferating T cells. (**C** and **D**) IFN-γ (**C**) and IL-10 (**D**) were measured by ELISA in 48- or 96-hour supernatants collected from cocultures of activated T cells with TANs or PBNs. Summary results from 8 lung cancer patients are shown in the graph (Wilcoxon matched-pairs rank test). (**E**) The percentage of IFN-γ– and IL-10–producing T cells cultured with TANs or PBNs was measured by intracellular cytokine staining at 48 hours of stimulation. The dot plots represent 1 of 3 independent experiments. Numbers represent the percentage of $CD4^+$ and $CD8^+$ cells.

PBMCs that had been stimulated with different concentrations of anti-CD3 Abs (Supplemental Figure 2B). Four days later, TANs dramatically increased the proliferation of $CD4^+$ T cells from 15% to 64% ($P < 0.01$) and that of $CD8^+$ T cells from 12% to 61% ($P < 0.01$). Moreover, the coculture of TANs with highly activated T cells (anti-CD3 Abs, 2.5 μg/ml) resulted in even more rapid division of these T cells.

Since the CD3/CD28–stimulated T cell response involves a robust polyclonal T cell proliferation, we investigated the ability of TANs and PBNs to modulate more physiological T cell responses induced by allogeneic DCs in a mixed-lymphocyte reaction. Allogeneic T cells (responders) were purified from the peripheral blood of healthy donors and cocultured with irradiated, mature, monocyte-derived DCs (MoDCs) (inducers) from unrelated donors. TANs or PBNs from patients with stage I–IIB lung cancer were added to the DCs as "third-party cells." Five days later, we found that inclusion of TANs resulted in an increased T cell proliferation that had been initiated by allogeneic MoDCs, compared with control experiments (Figure 5C). These experiments were repeated with TANs from 5 patients with early-stage lung cancer. By day 5, the addition of TANs increased the proliferation of T cells 1.7- to 2.8-fold compared with PBNs. The TANs did not appear to preferentially increase $CD4^+$ versus $CD8^+$ T cell expansion.

Next, we asked whether treatment of PBNs with TCM would recapitulate the ability of TANs to stimulate T cell proliferation. We exposed PBNs from healthy donors to a variety of TCMs collected from digested AC or SCC. The majority of these TCMs prolonged survival time of PBNs up to 48 hours (Figure 4B) and induced the expression of the activation marker CD54 on the surface of PBNs (Figure 2C). However, TCM-treated PBNs were not able to stimulate T cell proliferation to a significant level (Supplemental Figure 3H). This indicates that short-term exposure of mature PBNs to tumor-derived factors is not sufficient to convert PBNs into stimulatory cells.

In total, we analyzed the effect of TANs on the proliferation of T cells from 16 patients with lung cancer. Overall, TANs sig-

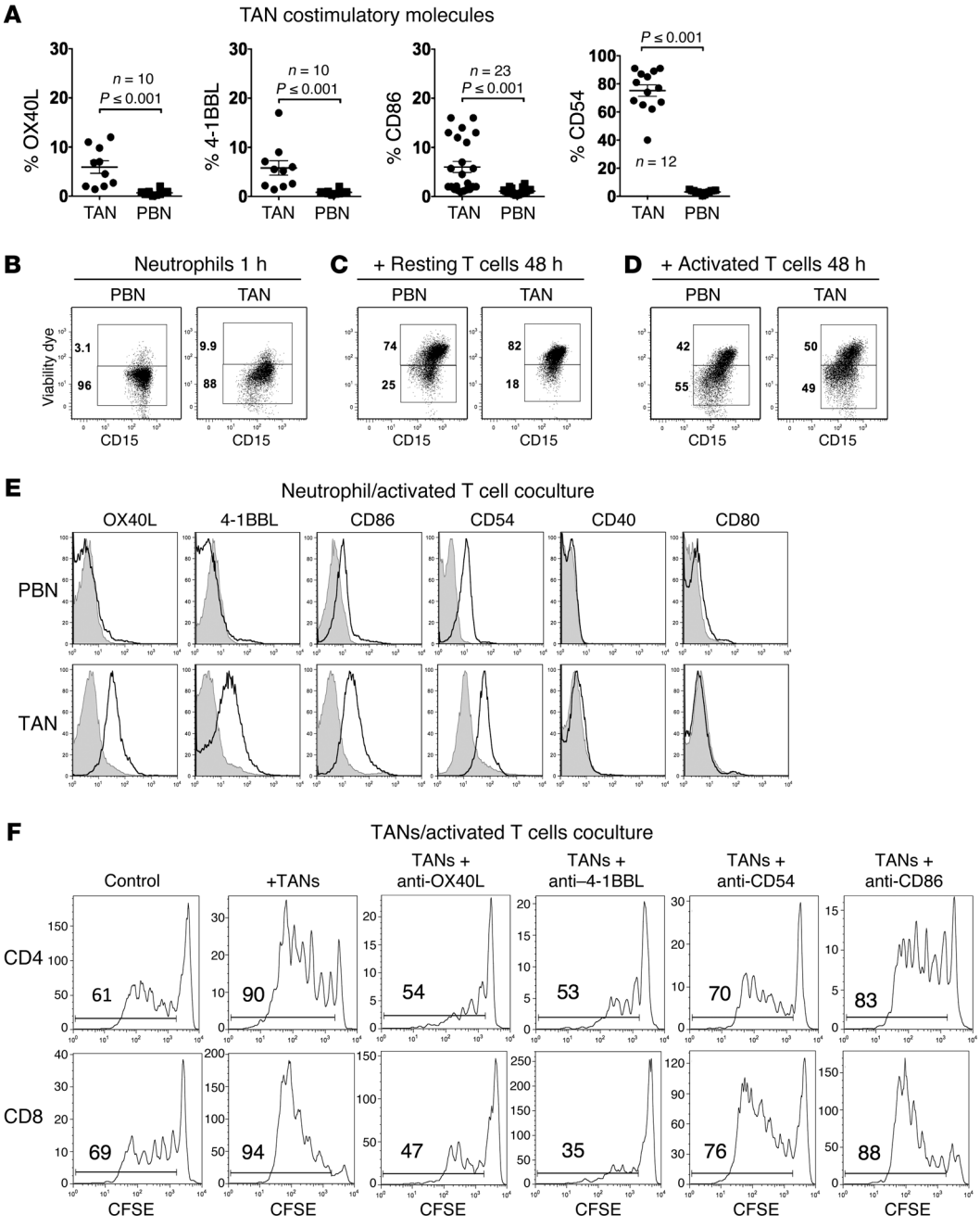

Figure 7. The expression of costimulatory molecules on TANs and their role in stimulation of T cell proliferation. (**A**) The expression of the costimulatory molecules on gated CD11b$^+$CD15^{hi} TANs and PBNs was analyzed by flow cytometry. The top panel summarizes the data for all the patients. Error bars represent mean ± SEM. Statistical analyses were performed with Student's *t* test for paired data. (**B**–**D**) Neutrophil survival in the cell culture. Zombie Yellow Fixable Viability dye was used to discriminate viable CD15 neutrophils cultured alone (**B**) or in the coculture with resting T cells (**C**) and CD3/CD28–activated T cells (**D**). Representative dot plots from 1 of 5 experiments are shown. For all dot plots, numbers represent the percentage of cells in each quadrant. (**E**) The expression of costimulatory molecules was analyzed by flow cytometry on gated live CD11b$^+$CD15^{hi} PBNs (top) and TANs (bottom) after 2 days of coculture with activated (black histograms) or resting autologous T cells (gray histograms). Results from 1 of 5 representative experiments are shown. (**F**) The efficacy of blocking Abs in ablating the stimulatory effect of TANs on T cell proliferation. Autologous PBMCs were stimulated with plate-bound anti-CD3 Abs and mixed with TANs at a 1:1 ratio in the presence or absence of blocking Abs against the indicated receptors for 4 days. Numbers on histograms represent the percentage of proliferating cells. Mouse IgG1 Abs were used as isotype control Abs in the control group. Results from 1 of 3 representative experiments are shown.

nificantly increased the proliferation of both CD4$^+$ and CD8$^+$ T cells an average of 2.1-fold (range 1.4- to 9-fold) compared with PBNs (Figure 5D; $P = 0.001$). Interestingly, as shown in Figure 5D and Supplemental Table 2, correlation analysis revealed that the majority of TANs from larger tumors were associated with a lower capacity to augment T cell proliferation than TANs from smaller tumors. We also divided lung cancer patients into 2 groups: patients with small tumors (<3 cm, $n = 7$) and patients with large tumors

(>3 cm, $n = 9$). The analysis of these 2 groups using a Mann-Whitney nonparametric test also revealed that stimulatory activity of TANs from small tumors was significantly higher than that of TANs from large tumors (Supplemental Table 2). Interestingly, there were no significant associations between stimulatory activity of TANs and histological type of tumor, tumor stage, and smoking history (Supplemental Table 2 and Supplemental Figure 4, A–C).

TANs enhance T cell activation. To further examine the cross-talk between activated T cells and neutrophils in lung cancer patients, we assayed T cell activation and the capacity of activated T cells to produce cytokines in the presence of TANs or PBNs. T cell activation was assessed within 24 hours of exposure to anti-CD3/anti-CD28 Abs. When T cells were cultured with TANs, they more markedly upregulated CD25 (65% ± 11% vs. 41% ± 17%, $P = 0.001$) and downregulated CD62L (39% ± 14% vs. 54% ± 11%, $P = 0.01$), compared with control T cell populations. A representative experiment depicted in Figure 6A (left and center columns) demonstrates the effect of TANs on the expression of these markers in activated T cells. TANs did not affect CD69 expression on activated T cells (data not shown). We also found that the percentage of activated $CD8^+$ cells expressing the lysosomal marker CD107a (LAMP-1) on the surface was twice as high in TAN/T cell cocultures compared with cultures of control T cells alone (Figure 6A, right column). Together, these data suggest that TANs tend to promote activation of T cells and degranulation of their cytotoxic granules.

The expression of CD25 also defines a distinct population of $CD4^+$ $FOXP3^+$ Tregs with suppressive activity in vitro and in vivo (42). However, after coculture for 4 days, analysis of FOXP3 expression showed no difference in activated $CD4^+$ cells cultured with or without TANs (4.5% ± 1.1% and 5% ± 1.5%, respectively, $P > 0.05$) (Supplemental Figure 3F).

We also determined whether TANs or PBNs modulated the production of key Th1 or Th2 cytokines in activated T cells. For these experiments, we mixed TANs or PBNs with autologous CD3/CD28–stimulated T cells purified from peripheral blood of patients with NSCLC and quantified IFN-γ and IL-10 in the supernatants 96 hours later. Figure 6C demonstrates that TANs significantly increased IFN-γ production by activated T cells, as TAN/T cell cocultures had a much higher concentration of IFN-γ compared with T cells cultured alone ($P = 0.02$). PBNs isolated from lung cancer patients did not affect the IFN-γ production by activated T cells. Intracellular staining revealed a significant increase in the frequency of IFN-γ–positive $CD4^+$ and $CD8^+$ cells in the coculture with TANs compared with activated T cells cultured alone (Figure 6E). While the percentage of IFN-γ–producing T cells was slightly changed, the stimulation of T cell proliferation by TANs resulted in a larger number of T cells and increased overall cytokine levels in the supernatant after 4 days of coculture. In addition, there was no significant difference in the production of IL-10 by activated T cells cultured with TANs or PBNs (Figure 6D). Intracellular IL-10 was not detected in activated T cells cultured in the presence or absence of TANs and PBNs (data not shown).

Direct cell contact between TANs and T cells is important for T cell stimulation. In order to understand the primary mechanism of TAN/T cell effects, we investigated whether direct cellular contact was necessary for TANs to stimulate T cell proliferation, using a transwell assay system that separated the TANs or PBNs from the T cells. The PBNs isolated from healthy donors or lung cancer patients did not induce the stimulation of T cell proliferation when the cells were mixed or separated (data not shown). When in direct contact, TANs induced a much higher level of T cell proliferation than in the transwell system, in which TANs were physically separated from activated T cells (Figure 6B). These data indicate that cellular contact is likely the chief mechanism by which TANs augment T cell proliferation. However, the TANs isolated from several patients demonstrated some stimulatory effect on T cells even when the cells were separated, suggesting that secreted factors are also involved in the stimulation of T cell proliferation, but to a lesser extent (Figure 6B, right column).

Expression of costimulatory molecules on TANs and their role in the stimulation of T cell proliferation. Given that the strong stimulatory effect of TANs on T cell proliferation was dependent on direct cell contact, we quantified the expression of costimulatory molecules (CD86, CD80, CD40, CD54 [ICAM-1], CD252 [OX40L], and CD137L [4-1BBL]) on the surface of TANs and PBNs by flow cytometry (Figure 7A). Circulating PBNs had minimal to no expression of these costimulatory molecules in all patients. The expression of CD54 was highly increased on the surface of TANs versus PBNs (75% ± 15% vs. 3% ± 1%, $P < 0.001$) (Figure 7A). In addition, we found moderate but statistically significant upregulation of CD86, OX40L, and 4-1BBL on the surface of TANs but not PBNs ($P < 0.001$). Figure 7A demonstrates that the expression of these markers varied widely from 0.5% to 20% among all cancer patients. However, the differences in the expression of these costimulatory molecules on TANs were not significantly correlated with tumor type, size, or stage (Supplemental Table 2 and Supplemental Figure 4, E–H). We were not able to detect the expression of CD80 or CD40 markers on the surface of TANs.

Given that TANs enhance the activation of T cells during cell coculture, we examined whether activated T cells, in turn, upregulate the expression of costimulatory molecules in TANs to further bolster their own proliferation. To test this hypothesis, we activated T cells with plate-bound anti–CD3/CD28 Abs and mixed them with autologous TANs or PBNs. Two days later, flow cytometry was used to characterize the viability of neutrophils cocultured with activated T cells and expression of costimulatory molecules on gated live $CD11b^+CD15^+$ TANs. Flow cytometry revealed that in the presence of activated T cells, the TANs and PBNs survived longer than neutrophils cultured with resting T cells (Figure 7, B–D). The activated T cells increased the lifespan of TANs and PBNs to 4 days (Supplemental Figure 3G). Importantly, TANs cocultured with activated T cells markedly upregulated OX40L, 4-1BBL, CD54, and CD86 costimulatory molecules, whereas PBNs increased expression of only CD86 and CD54 (Figure 7E). These data suggest that a preexisting activated state of TANs or some enhanced plasticity is required for subsequent T cell–induced upregulation of these costimulatory molecules. CD80 and CD40 continued to show low levels of expression on PBNs and TANs following exposure to activated T cells.

Next, we investigated the functional significance of these costimulatory molecules. In 3 experiments, TANs and CFSE-labeled activated autologous T cells were cocultured in the presence of blocking Abs against these upregulated costimulatory molecules. Figure 7F shows a representative experiment (all 3 showed the same results)

where the stimulatory effect of TANs was partially abrogated in the presence of anti-CD54 and -CD86 blocking Abs (right columns). The most pronounced effect was observed when anti-OX40L or anti–4-1BBL blocking Ab was added to the T cell/TAN coculture. These Abs completely blocked the strong stimulatory activity of TANs (Figure 7F, center columns). Notably, in the control groups, the proliferation of T cells without TANs was not affected by the presence of any of these blocking Abs. These data suggest that TANs enhance T cell proliferation by direct cell-cell signaling, likely due to the OX40L/OX40 and 4-1BBL/4-1BB pathways. Both pathways appear equally important for T cell proliferation.

Taken together, these results suggest that there is ongoing cross-talk between activated T cells and TANs that results in dramatic upregulation of costimulatory molecules on the surface of TANs, which enhances T cell proliferation. This interaction between the innate and adaptive sides of the immune system requires direct cell-cell interactions due to receptor engagement, although secretory cytokines may have a limited role in this relationship.

Discussion

This study provides a comprehensive phenotypic and functional characterization of tumor-infiltrating neutrophils in early-stage lung cancer patients. Our key observations were that TANs represented a significant proportion of the cellular composition of human lung tumors and that, in contrast to our expectations, early-stage lung cancer TANs were not hypofunctional or immunosuppressive, but were able to stimulate T cell responses.

Our data show that TANs express a "classic" activated phenotype characterized by upregulation of the adhesion molecule CD54 (ICAM-1) and downregulation of CD62L (L-selectin), CXCR1, CXCR2, and CD16 (29, 30). Another major change in the infiltrating neutrophils compared with systemic PBNs was in chemokine receptor expression, including upregulation of CCR5, CCR7, CXCR3, and CXCR4, and downregulation of CXCR1 and CXCR2. It has been suggested that the acquisition of new chemokine receptors by neutrophils at inflammatory sites expands their functional profile (33); however, the exact role of the chemokine receptors expressed on TANs is still unknown, and further studies are required to understand their functional significance.

In this study, we also characterized the phenotype of neutrophils from the nonmalignant lung tissue to demonstrate which characteristics of TANs are specific for the tumor microenvironment and which simply reflect the differences between blood and lung tissue neutrophils. Neutrophils from distant tissue versus TANs were more similar to each other than to blood neutrophils. However, we found that TANs exhibit an even more activated phenotype compared with neutrophils isolated from "distant" noninvolved lung tissue. The level of CCR5, CCR7, CXCR3, and CXCR4 expression on distant neutrophils was intermediate between those of PBNs and TANs. Although these data are interesting, there are some caveats associated with comparison of neutrophils from tumor and distant lung tissue. First, we believe the surrounding nonmalignant lung tissue is likely to be influenced by the adjacent tumor, so neutrophils infiltrating the adjacent lung tissue may not have exactly the same function and phenotype as those infiltrating normal lung tissue. Second, compared with tumor-infiltrating neutrophils, the majority of lung neutrophils likely represent a pool of marginated neutrophils (cells that are adherent to the endothelium of blood vessels of lung tissue) and neutrophils from alveolar space, making them difficult to define as tissue-specific.

In addition to changes in activation, we also found that the tumor microenvironment stabilizes and prolongs the survival of infiltrating neutrophils. In the presence of tumor-conditioned medium (TCM) rich in proinflammatory factors, such as IFN-γ, IL-6, IL-8, and GM-CSF (Figure 4B), TANs and naive blood neutrophils developed a significant survival advantage compared with control neutrophils. This is likely due to the ability of these proinflammatory factors to prolong the lifespan of human neutrophils by delaying apoptosis (43, 44).

Once TANs are activated in the tumor microenvironment, they appear to add to the complexity of the inflammatory milieu and are likely involved in the attraction of other leukocytes. TANs secreted large quantities of IL-8 in cell culture, which has been found to self-promote neutrophil survival and recruit more neutrophils. We also found that TANs released various immunoregulatory cytokines, chemokines, and growth factors, such as the proinflammatory mediators CCL2 (MCP-1), IL-8, CCL3 (MIP-1α), and IL-6, as well as the antiinflammatory cytokine IL-1RA. On the other hand, TANs can secrete factors that could be protumorigenic. MIP-1α may act as a growth, survival, and chemotactic factor for tumor cells (45). In our study, we did not see high levels of proangiogenic VEGF, but there were other growth factors that might support angiogenesis, such as FGF, HGF, and EGF.

Over the last decade, there has been an increasing focus on the interactions between myeloid cells and T cells in tumor-bearing mice. Most of these studies have focused on MDSCs and TAMs. The vast majority of the data suggest that these cells inhibit T cell proliferation and function (46–49). With regard to TAMs, the current paradigm is that these cells are primarily tumor-promoting (M2-type) cells but, under certain conditions, can be reprogrammed into tumor-inhibitory (M1-type) cells with therapeutic potential (50–52). Much less is known about murine TANs; however, work by our group (8) and others (5, 53–55) suggests that a similar N1 (antitumor) and N2 (protumor) polarization exists, and that most advanced tumors harbor N2-like TANs. Given this framework, we anticipated human TANs would inhibit T cell responses in human lung tumors. Unexpectedly, however, freshly isolated TANs from early-stage lung cancer patients did not suppress IFN-γ production or proliferation of T cells that had been activated with anti–CD3/CD28 Abs or allogeneic DCs. Instead, TANs increased T cell IFN-γ production and activation, and dramatically amplified T cell proliferation. Direct cell-cell contact was important for the neutrophil-mediated stimulation of T cell proliferation. One important feature of this interaction was cross-talk and mutual cell activation. With coculture, T cells further upregulated activation markers and produced more IFN-γ, whereas TANs upregulated the costimulatory molecules CD86, CD54, OX40L, and 4-1BBL. These molecules are not constitutively expressed on the surface of circulating neutrophils; however, they can be rapidly translocated from cytoplasmic granules onto the surface of neutrophils or be synthesized de novo under the appropriate circumstances (19, 56).

Follow-up experiments using blocking Abs against various costimulatory molecules showed that the OX40L/OX40 and 4-1BBL/4-1BB pathways were critical in TAN-mediated augmen-

tation of T cell proliferation. 4-1BBL/4-1BB and OX40/OX40L represent a pair of costimulatory molecules critical for T cell proliferation, survival, cytokine production, and memory cell generation, as well as reverse signaling for further activation of APCs (57, 58). Typically, the costimulatory molecules 4-1BBL and OX40L are expressed on APCs, including mature DCs, activated macrophages, and B cells (57). Our data suggest that the 4-1BBL and OX40L costimulatory molecules can also be upregulated on activated TANs as a result of the interaction with activated T cells. Thus, the OX40L/OX40 and 4-1BBL/4-1BB pathways have the potential to enhance antitumor immunity and break tumor-induced immune suppression and immunological tolerance. Furthermore, costimulation through 4-1BBL/4-1BB protects T cells from activation-induced cell death and enhances the antitumor effector functions of $CD8^+$ melanoma tumor–infiltrating lymphocytes (59, 60).

Our data are consistent with previous studies showing that granulocytes can provide accessory signals for T cell activation (19, 21, 22). For instance, Radsak et al. reported that human circulating neutrophils are accessory cells for T cell activation after treatment with IFN-γ and GM-CSF (19). They found that neutrophil-dependent T cell proliferation could be partially inhibited by blocking Abs against MHC class II, CD86, and CD54. Interestingly, our findings showed that lung tumors were able to produce IFN-γ and GM-CSF, as well as to induce expression of CD86 and CD54 costimulatory molecules in TANs. However, blocking CD86 did not substantially inhibit the stimulatory capacity of TANs compared with blocking OX40L or 4-1BBL. Inhibiting CD54 resulted in partial ablation of this effect.

Our data are consistent with some literature showing the antitumor potential of neutrophils during tumor growth in some models (61–63). For instance, Suttmann and colleagues demonstrated that polymorphonuclear neutrophils are an indispensable subset of immunoregulatory cells and orchestrate T cell chemotaxis to the bladder during bacillus Calmette-Guérin immunotherapy (64). Augmentation of T cell proliferation and/or survival by tumor-infiltrating neutrophils was found to be critical in the establishment of antitumor immunity following photodynamic therapy (65). Our group found that the blockage of TGF-β could convert N2 TANs to N1 TANs in murine models of mesothelioma and lung cancer (8).

Can human TANs exert antitumor (N1-like) activity? Although TANs isolated from early-stage lung cancers resemble murine antitumor N1 TANs, our data suggest that as tumors become larger, they become less stimulatory. It is thus possible that TANs from even more advanced tumors may become frankly protumorigenic. This concept, with regard to TANs, has recently been described in a murine tumor model. Mishalian et al. reported that TANs from early tumors were cytotoxic to tumor cells and produced higher levels of TNF-α, NO, and H_2O_2 compared with TANs in larger, established tumors (66). We are trying to test this "myeloid cell immunoediting" hypothesis with TANs from patients with advanced lung cancer (stages III and IV); however, this is logistically challenging since these individuals do not routinely undergo tumor resection and are managed with chemotherapy and radiation therapy.

Our study provides several explanations for the inconsistent data in the literature with regard to prognostic implications of TANs in cancer patients (11, 14–17). To date, most clinical studies have used immunohistochemical analyses of tumors to correlate the presence of granulocytes with prognosis. However, this approach is unable to assess the phenotype of these cells. In this study, we have demonstrated there is a heterogeneous expression of surface receptors on TANs. Thus, subpopulations of TANs likely exist in tumors at different stages of disease development and perform different functions. Also, since TANs might lose or change their antitumoral functions as the tumors progress, a simple neutrophil count in tumor tissue at any one time point (where the pro- versus antitumor status of the neutrophils is not known) may not be an accurate parameter for clinical prognosis.

In summary, our findings characterize tumor-infiltrating neutrophils in patients with lung cancer for the first time. Although the presence of a minor suppressive subpopulation of TANs cannot be excluded, our data suggest that TANs do not significantly contribute to inhibition of T cell responses in patients with early-stage lung cancer. Rather, the majority of neutrophils recruited into the tumor microenvironment undergo phenotypic and functional changes that result in the formation of cells that could potentially augment and support T cell responses. However, the in vitro conditions necessary for our experiments may not necessarily reflect what actually transpires in vivo. In addition, the ability of TANs to augment T cell responses is only one of many potential characteristics of antitumoral N1 neutrophils and does not entirely define TANs as antitumoral cells. In these studies, we were not able to assess the role of human TANs in the regulation of tumor cell proliferation, matrix remodeling, angiogenesis, and metastasis. Areas of future investigation in our laboratory are focused on deciphering subpopulations of neutrophils in human lung cancers and further characterization of the role of TANs in the regulation of tumor development using in vivo models. Ultimately, these findings may have important clinical implications, such as ways to take advantage of the T cell stimulatory activity of TANs and boost the efficacy of vaccines based on cytotoxic T lymphocyte induction.

Methods

Study design

A total of 86 patients with stage I–II lung cancer, who were scheduled for surgical resection, consented to the harvest of a portion of their tumor and blood for research purposes. All patients signed an informed consent document that was approved by the University of Pennsylvania Institutional Review Board, and met the following criteria: (a) histologically confirmed pulmonary squamous cell carcinoma (SCC) or adenocarcinoma (AC), (b) no prior chemotherapy or radiation therapy within 2 years, and (c) no other malignancy. Detailed characteristics of the patients can be found in Supplemental Table 1.

Reagents

The enzymatic cocktail for tumor digestion consisted of serum-free Hyclone Leibovitz L-15 medium supplemented with 1% penicillin-streptomycin, collagenase type I and IV (170 mg/l = 45–60 U/ml), collagenase type II (56 mg/l = 15–20 U/ml), DNase I (25 mg/l), and elastase (25 mg/l) (all from Worthington Biochemical). Cell culture reagents are described in Supplemental Methods.

Lymphocyte isolation from peripheral blood

Standard approaches were used. See Supplemental Methods.

Preparation of a single-cell suspension from lung tumor tissue

Surgically removed fresh lung tumors from patients were processed within 20 minutes of removal from the patient. In brief, the tumors were trimmed, sliced into small pieces, and digested for 1 hour at 37°C with shaking. After rbc lysis, cell viability was determined by trypan blue exclusion or Fixable Viability Dye eFluor 450 staining (Supplemental Figure 1B). If the viability of cells was less than 80%, dead cells were eliminated using a Dead Cell Removal Kit (Miltenyi Biotec Inc.). See Supplemental Methods for full details.

Tumor-conditioned medium

See Supplemental Methods.

Neutrophil isolation

Since temperature gradients can activate neutrophils, all tissues and reagents were maintained at a constant temperature during preparation. After tumor harvest, TANs and PBNs were prepared at room temperature and rapidly used.

TANs. A single-cell suspension was obtained by enzymatic digestion of tumor tissue. TANs were isolated from tumor cell suspensions using positive selection of $CD15^+$ or $CD66b^+$ cells with microbeads according to the manufacturer's instructions (Miltenyi Biotec Inc.). In some experiments, TANs were isolated by flow cytometric cell sorting based on the phenotype of TANs as $CD45^+CD11b^+CD66b^+CD15^+$. Sterile cell sorting was performed on the BD FACSAria II (BD Biosciences). For more details see Supplemental Methods.

PBNs. EDTA-anticoagulated peripheral blood was collected from lung cancer patients during surgery or from healthy donors, and density-gradient centrifugation was performed. To account for any possible effect of tissue digestion enzymes on neutrophil function, peripheral blood granulocytes were processed in a similar manner.

The purity and activation status of isolated TANs and PBNs were measured by flow cytometry for the granulocyte/myeloid markers CD66b, CD15, arginase-1 (Arg1), myeloperoxidase (MPO), and CD11b, and the activation markers CD62L and CD54. The TANs demonstrated high cell viability with minimal enzyme-induced premature cellular activation or cleavage of myeloid cell markers (Supplemental Figure 1). The purity of TANs and PBNs was typically higher than 94%. Isolates with less than 90% purity were discarded.

Flow cytometry

Flow cytometric analysis was performed according to standard protocols. Details about the Abs used are listed in Figure 3A. Matched-isotype Abs were used as controls. For more details see Supplemental Methods.

T cell proliferation assay

T cell proliferation induced by plate-bound anti–human CD3 (clone: OKT3) and/or anti-CD28 (clone: CD28.2) Abs was assessed using standard CFSE dilution methods. PBMCs or purified T cells (responders) were labeled with CFSE and cocultured in CD3/CD28–coated plates for 4 days in the complete cell culture medium. The CFSE signal was analyzed by flow cytometry on gated $CD4^+$ or $CD8^+$ lymphocytes. In several experiments, blocking Abs against CD86 (clone: IT2.2), CD80 (clone: 2D10), OX40L (clone: 11C3.1), 4-1BBL (clone: 5F4), CD54 (clone: HCD54), or CD40 (clone: 5C3) (all from Biolegend) were added to the cocultures of TANs and activated T cells at the concentration 1 μg/ml. In other experiments, the proliferation of T cells was assessed by flow cytometry using the BrdU Flow Kit (BD Pharmingen). For more details see Supplemental Methods.

Allogeneic mixed lymphocyte reaction

Purified allogeneic T cells from healthy donor PBMCs were used as responders and reacted with irradiated, mature, monocyte-derived DCs (MoDCs) (inducers) from unrelated healthy donors. Immature MoDCs were prepared by culturing of adherent peripheral blood monocytes for 7 days in DMEM supplemented with 10% FBS, recombinant human GM-CSF (50 ng/ml), and IL-4 (50 ng/ml). To mature the MoDCs, LPS (100 ng/ml) was added to the cell culture for 24 hours before harvesting. The TANs or PBNs (regulators) were added to the DC-induced mixed lymphocyte reaction as "third-party cells" at a ratio of 1:0.25:1 (regulator/inducer/responder). Five days later, the proliferation of $CD4^+$ and $CD8^+$ T cells was measured using flow cytometric analysis of CFSE dilution.

Phagocytosis

The phagocytic activity of TANs and PBNs was assayed with the pHrodo Red *E. coli* BioParticles Phagocytosis Kit for flow cytometry (Life Technologies), according to the manufacturer's instructions.

Chemotaxis

We used a previously established protocol for fluorescence-based measurement of neutrophil migration in vitro across a polycarbonate filter (67) with minor modifications. See Supplemental Methods for details.

Neutrophil survival

Freshly isolated TANs or PBNs were cultured in complete cell culture medium in the presence or absence of 50% v/v of TCM for 20 hours. Neutrophil viability, apoptosis, and necrosis were measured using the FITC-Annexin V Apoptosis Detection Kit (Biolegend) and analyzed by flow cytometry, according to the manufacturer's instructions.

Measurement of ROS

The production of H_2O_2 in TANs and PBNs isolated from lung cancer patients and healthy donors was measured using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen), according to the manufacturer's instructions. See Supplemental Methods for details.

Measurement of cytokines, chemokines, and growth factors

Single-cell suspensions were obtained from lung tumors by enzymatic digestion, as described above. TANs and PBNs were isolated from lung cancer patients, as described above. Both unseparated cells and isolated neutrophils from digested tumors, and PBNs, were resuspended in DME/F-12 1:1 medium with 10% FBS at a concentration of 1×10^6 cells/ml. Twenty-four hours later, cell culture supernatants were collected, filtered, and stored at –80°C until measurement. The levels of 30 cytokines/chemokines and growth factors were measured using the Cytokine Human Magnetic 30-Plex Panel for the Luminex platform (Invitrogen). The production of IFN-γ, IL-10, and GM-CSF was measured with commercial ELISA kits purchased from BD Bioscience.

Immunohistochemistry

The tumor microarrays (TMAs) were constructed from formalin-fixed, paraffin-embedded tumor and adjacent normal specimens collected at the time of surgical resection. Sections from 45 AC and 25 SCC patients were analyzed. After standard antigen retrieval, the TMAs were double-

stained with an anti-cytokeratin Ab to label cancer cells and an Ab against human MPO to label neutrophils. Slide imaging was performed on a Vectra automated imaging robot and analyzed using Inform analysis software. Data are expressed as the intraepithelial or stromal hematopoietic cell density per square millimeter of tumor tissue. In addition, we costained for neutrophils (MPO), APCs (HLA-DR), and T cells (CD3), using their respective Abs. See Supplemental Methods for details.

Statistics

All data were tested for normal distribution of variables. Comparisons between 2 groups were assessed with a 2-tailed Student's *t* test for paired and unpaired data if data were normally distributed. Nonparametric Wilcoxon matched-pairs test and Mann-Whitney unpaired test were used when the populations were not normally distributed. Likewise, multiple groups were analyzed by 1-way ANOVA with corresponding Tukey's multiple comparison test if normally distributed, or the Kruskal-Wallis with Dunn's multiple comparison test if not. Nonparametric Spearman test was used for correlation analysis. All statistical analyses were performed with GraphPad Prism 6. A *P* value less than 0.05 was considered statistically significant.

Study approval

The study was approved by the University of Pennsylvania Institutional Review Board (IRB no. 813004). All patients signed an informed consent document.

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